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DIFFERENTIATION OF OXYHEMOGLOBINS BY MEANS OF MUTUAL SOLUBILITY TESTS.

By KARL LANDSTEINER and MICHAEL HEIDELBERGER.

(From the Laboratories and the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, July 6, 1923.)

In recent papers[1] the authors were able to show by serological tests that hemoglobin exhibits species specificity to a high degree. It seemed of advantage, however, to demonstrate these differences, undoubtedly chemical in character, by some independent method.

The solubility of an individual protein itself might be taken as a criterion of chemical entity, as has been suggested recently by Cohn.[2] As, however, this method requires the attainment of absolute purity, aside from the possibility that several substances might have the same solubility within the limits of error, another method was sought.

Of the possibilities which suggested themselves, a promising one seemed the use of the well known fact that the solubilities of substances which do not react with each other are additive. As a consequence it would be expected that if two samples of oxyhemoglobin were different each would dissolve in a saturated solution of the other as in pure water itself. On the other hand, if two preparations were identical, a saturated solution of one would obviously be saturated to both.

As the method used in the present work was a comparative one, extreme accuracy was not aimed at. Hence the solubility determinations were carried out simply at room temperature. 1 cc. samples of the solutions were used, and the content in hemoglobin was determined by drying to constant weight *in vacuo* at 40–50°C.

[1] Landsteiner, K., *Verslog Kon. Acad. van Wetensch. te Amsterdam*, 1921, xxix, 1029. Heidelberger, M., and Landsteiner, K., *J. Exp. Med.*, 1923, xxxviii, 561.

[2] Cohn, E. J., *J. Gen. Physiol.*, 1921–22, iv, 697.

The preparations of oxyhemoglobin were made according to the method recently published by one of us.[3] In each case freedom from salts was controlled by conductivity measurements, and in several instances electrometric pH determinations were run on the solutions in order to make certain that the observed solubility effects were not due to differences in the pH of the solutions.

As an example of the manipulation used the following experiment (No. 2) is described.

Recrystallized horse and dog oxyhemoglobin were used. The conductivities of the aqueous solutions were respectively 4.3×10^{-5} and 2.7×10^{-5}. Each oxyhemoglobin in the form of a moist crystalline paste was mixed with water in a tube and shaken mechanically for $\frac{3}{4}$ of an hour. That equilibrium was approximately attained within this period was shown by a determination of the hemoglobin in solution, and a comparison of the amount found with the hemoglobin content after the next shaking, the latter value being the one given at the head of each column in the tables. Usually a slight increase was noted. At the end of the period of shaking the tubes were centrifuged and the supernatant liquid from each was poured in equal parts into two tubes. To one of these was added more of the homologous protein, while to the other the oxyhemoglobin of the other species was added. The four tubes were now shaken again for $\frac{1}{2}$ hour and centrifuged. In every case before taking the analytical samples the solutions were filtered through small analytical filters in order to hold back any crystal fragments. 1 cc. portions of each were now dried and the residue was weighed.

The results are given in the following tabulations, in which the figures represent the weight of the residue from 1 cc. in gm. In each case the value after "difference" should approximate that at the head of the opposite column.

Experiment 1.

Dog + dog	0.0275	Horse + horse	0.0080
Dog + horse	0.0384	Horse + dog	0.0302
Difference	0.0109	Difference	0.0222

[3] Heidelberger, M., *J. Biol. Chem.*, 1922, liii, 31.

Experiment 2.

Conductivities: horse HbO_2 4.3×10^{-5}; dog HbO_2 2.7×10^{-5}.

Dog + dog	0.0238	Horse + horse	0.0158
Dog + horse	0.0372	Horse + dog	0.0417
Difference	0.0134	Difference	0.0259

Experiment 3.

Dog + dog	0.0260	Horse + horse	0.0151
Dog + horse	0.0395	Horse + dog	0.0400
Difference	0.0135	Difference	0.0249

Experiment 4.

Conductivities: horse HbO_2 3.2×10^{-5}; dog HbO_2 3.5×10^{-5}; guinea pig HbO_2 2.9×10^{-5}.

pH electrometric;* respectively: 6.69, 6.82, 6.76.

Dog + dog	0.0256	Horse + horse	0.0107
Dog + horse	0.0356	Horse + dog	0.0293
Difference	0.0100	Difference	0.0186
Dog + dog	0.0256	Guinea pig + guinea pig	0.0046
Dog + guinea pig	0.0274	Guinea pig + dog	0.0318
Difference	0.0018†	Difference	0.0272
Horse + horse	0.0107	Guinea pig + guinea pig	0.0046
Horse + guinea pig	0.0140	Guinea pig + horse	0.0159
Difference	0.0033	Difference	0.0113

*The writers are indebted to Dr. A. B. Hastings for these determinations.

†That this value is too low is probably due to the fact that, as mentioned above, saturation was not always complete at the end of the first shaking. This would have little influence except in a case such as this, in which the solubility of the second type of hemoglobin is much less than that of the first.

Experiment 5.

Conductivities: guinea pig 2.8×10^{-5}; rat 3.8×10^{-5}.

pH electrometric, respectively: 6.69, 6.95.

Guinea pig + guinea pig	0.0040	Rat + rat	0.0039
Guinea pig + rat	0.0071	Rat + guinea pig	0.0086
Difference	0.0031	Difference	0.0047

It will be seen from the above experiments that in four of the species tested, differences between the oxyhemoglobins were indicated by the increase in the dissolved hemoglobin. This increase was in most cases not far from the solubility of the added type of oxyhemoglobin in water alone. It thus appears that the method is capable of bringing to light chemical differences between the oxyhemoglobins of not too closely related species. The trial of this procedure in the study of other proteins would therefore seem desirable.

As a control on the method Experiment 6 was performed. In this experiment two different lots of horse oxyhemoglobin were compared as before. As was to be expected the result was negative.

Experiment 6.

Comparison of two different preparations of horse oxyhemoglobin (I and II). Conductivities: I 2.2×10^{-5}; II 3.3×10^{-5}.

I + I	0.0120	II + II	0.0107
I + II	0.0118	II + I	0.0107

Experiment 7.

Conductivities: horse HbO_2 3.3×10^{-5}; donkey 1.5×10^{-5}.

Horse + horse	0.0166	Donkey + donkey	0.0086
Horse + donkey	0.0139	Donkey + horse	0.0128
Difference	−0.0027	Difference	0.0042

In Experiment 7 oxyhemoglobins of two closely related species, namely the horse and the donkey, were investigated. This case was of special interest in that our serological tests indicated so slight a dissimilarity as to render difficult a serological differentiation. In the present series of experiments, also, this case differed from the others, as no addition of solubilities took place. While it would seem that the final values were between the solubilities of the two preparations, too much stress cannot be laid upon this point, as the absolute value of the solubility of horse oxyhemoglobin varied in the different experiments, and the cause of this variation has still to be determined.

Since, from general considerations, identity of the two proteins is improbable, it would appear that the two oxyhemoglobins behave

as isomorphous compounds. It is interesting that such a relationship should be found in a case in which the proteins appear compatible *in vivo*, as is shown by the ability to cross these animals. In this connection we must mention the comprehensive work of Reichert and Brown[4] on the crystallography of hemoglobin with respect to species differences. Its possible bearing upon the point just mentioned has been stated by Loeb.[5]

CONCLUSIONS.

1. The rule of addition of solubilities is applicable to the differentiation of the oxyhemoglobins of not too closely related species.

2. The oxyhemoglobins of the horse, dog, rat, and guinea pig show differences when tested by this method. The oxyhemoglobins of the donkey and horse show a similarity which is best explained by the assumption of isomorphism.

[4] Reichert, E. T., and Brown, A. P., *Carnegie Inst. Washington, Pub. 116*, 1909.
[5] Loeb, J., *Science*, 1917, xlv, 191.

INFLUENCE OF LIGHT OF VERY LOW INTENSITY ON PHOTOTROPIC REACTIONS OF ANIMALS.*

By H. KEFFER HARTLINE.

(From the Biological Laboratory of Lafayette College, Easton.)

(Received for publication, August 20, 1923.)

INTRODUCTION.

Animals exhibiting phototropic reactions often orient equally strongly to light throughout a wide range of intensity. However, every stimulus to which an animal responds has a threshold of intensity below which that stimulus fails to elicit any response from the animal. It is therefore important to study the reactions of animals to stimuli which in intensity are in the region of this threshold in order to determine the nature and extent of the response.

The animals used in the investigation of these features were land isopods, which exhibit strong negative phototropism. These forms have been studied by Torrey and Hays (1914) and Cole (1907), but the most extensive work is that by Abbott (1918–19), who made a comparative study of several species, and found that they were equally negative in turning away from a light source in illuminations ranging from several hundred meter candles to 0.01 meter candle. Obviously the threshold of stimulation must lie at a very low value. It has been the object of this work to determine this threshold and to observe the nature of the responses to light in the region of this intensity.

Method.

The isopods were collected in the immediate vicinity of Easton, Pennsylvania. No accurate identification was attempted, but with

* This work was done under the direction and personal supervision of Dr. B. W. Kunkel of Lafayette College. I am indebted to him for much stimulating advice and helpful criticism at every step of the way, as well as for placing at my disposal the facilities of the Jenks Biological Laboratory.

the assistance of Dr. Kunkel the following forms were recognized: *Oniscus asellus* L., *Porcellio scaber* Latreille, *Porcellio rathkei* Brandt, *Armadillidium vulgare* Latreille. These were kept in a healthy condition for several months confined in a covered glass dish containing moist loam, dead leaves, and rotting wood. Individuals used in experiments were separately confined in small glass vials, each containing a bit of loam and leaf stuff, and stoppered with cotton. The vials were set in the dish and the whole kept in a dark cupboard.

The method of studying the responses of these animals was essentially the same as that used by Abbott (1918–19). The isopod was placed on a dead-black table top in the center of a circle 10 cm. in radius, and stimulated by horizontal illumination. The point on the circumference at which the animal left the circle was recorded. Several such responses were obtained from each animal, and several animals were used for each intensity of light.

Two factors should be considered in providing a field of illumination. First, since the intensity gradient of a given path rapidly changes with change in the distance from the source, and since Cole's work (1907) indicates that the angle subtended by the light source may influence the response, it is necessary to find some way of changing the illumination other than by changing the distance of the animal from the source.

The field of illumination in which the animals were placed was obtained by means of the very small image of a Mazda filament, formed by a converging lens. This image, which was a fine, very bright horizontal line 1 cm. long, served practically as a point source, and gave a wide, even field. At the point where this image was formed a diaphragm was arranged with an aperture 1 cm. by 1 mm., which cut off all the rays that were not focused at that point. To eliminate the unevenness caused by the cheap lens, a diffusing screen, consisting of a piece of frosted glass, was inserted between the lamp and the lens. Placing it here instead of at the slit in the diaphragm eliminated the side area of weak illumination, and made it possible, by moving the screen closer to the lens, to decrease the intensity of the image. By inserting another such screen and two light blackened photographic plates in various combinations and positions, an unbroken range of intensity could be obtained, from 0.228 to

0.000005 c.p. This furnished a method of changing the illumination on any given point without changing the distance from the image to that point. A large number of determinations of the intensity of the image for various screens at different positions were made with a Lummer-Brodhun photometer, use of which was obtained through the kindness of the Electrical Engineering Department of Lafayette College. Measurement of the low intensities was very difficult, and involved considerable error. The extent of the error could, however, be determined by setting the photometer distinctly off balance on first one side and then the other. Lamp, lens, and screens were enclosed in a light-proof box, the diaphragm forming one end, and allowing the light to shine on the experiment table. The illumination at the center of the circle was calculated (in meter candles) in the usual way. On the whole, this scheme proved highly satisfactory, and, except for the labor of making a large number of measurements, is to be recommended where a wide, even field of illumination is required, and where it is necessary not to change the distance from the source of light to the point.

The experiment table was covered with dead-black cloth which is often used in making blackboards. This gave an even surface which was rough enough so that the animals could walk with ease. A circle 10 cm. in radius was drawn in chalk with its center 39 cm. from the source of light. Two diameters, one coinciding with the axis of the cone of light, and the other at right angles to it, referred to as the zero line, were drawn lightly. The circumference of the circle was marked off, in divisions of 5° each, in both directions from the zero line to the axis on each side. The quadrants toward the light are referred to as positive, those away from the light as negative. The quadrants were numbered, and the thirty degree divisions indicated, so that any point could be quickly determined at a glance in degrees + or − from the zero line. The box containing the light was set up so as to give horizontal illumination over the circle, the slit being 5 mm. above the table top.

The critical illuminations to be worked with would obviously be too low to make it possible for the experimenter to see the animal. At Dr. Kunkel's suggestion, I experimented with luminous material, and found that small chips could be glued to the back of the isopod without

interfering with its movements. By this means, together with the use of luminous index points on the experiment table, the responses of the animals could be observed in total darkness and in extremely low illuminations. Three luminous index points were arranged so as to be visible from above but invisible to the animal on the table. The luminous chips which were placed on the animals were made by mixing powdered luminous material (Hammer Radium Company, Denver, Colorado) with thin collodion, and spreading the paste on tissue paper. When dry, the under side was blackened with India ink, and small chips (2 mm. by 1 mm.) were cut out. These were tough and

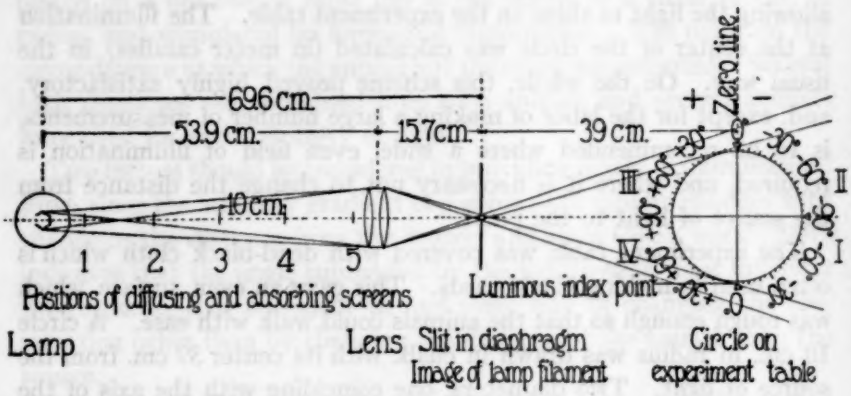

FIG. 1. Plan of apparatus. Source of illumination is image of Mazda filament made by lens. Diaphragm cuts out all other rays. Lamp, lens, and screens (diffusing and absorbing) enclosed in a light-proof box. Animals subjected to lateral illumination at the center of the circle on the experiment table.

durable, and were glued on the fourth thoracic segment by a very small drop of freshly prepared (unpoisoned) gum arabic solution. The movements of the animal could be readily seen, and when the circumference was crossed, a flashlight was used to make the reading. A wet piece of absorbent cotton would quickly remove the luminous chip after an experiment.

Cardboard frames were used to shove the animal into position in the center of the circle. When the frame was lifted, the animal was freed and at the same time exposed to light.

The apparatus was set up in a photographic dark room, the walls of which were painted dead-black to prevent reflection. The room was generally quite dry and hot, which must be noted because of the sensitiveness of land isopods to these conditions.

Procedure.

It is clear that an animal placed in the center of a circle will, in the absence of any directive stimulus, tend to move out of the circle on the diameter along which it is headed. Certainly, any deviations in its path which arise as the result of obscure physicochemical processes set up by either internal stimuli or uncontrollable external stimuli will be purely chance, and will be such that the average of all such deviations to one side or the other will be at the diameter along which the animal is started. This is especially true if the records are taken alternately in opposite directions to eliminate asymmetry. Thus the average of a number of responses of an isopod facing along the zero line (alternately in each direction) should, in the absence of any directive stimulus, be 0°. If rays of light, to which they are negative, strike the isopods from the + side, they will tend to move away from the light in a curved path, so that the average of a number of responses will be a certain number of degrees in the negative region. The same uncontrollable variations will be present and will affect the path in the same chance way, but in this case the distribution will be heavily skewed toward the negative side. The angle that this average path under the directive action of light makes with the average path in darkness (0°) can be used as a measure of the phototropism of the animal.

An isopod was removed from its bottle, a luminous chip glued on its back (fourth thoracic segment) and the animal placed on the experiment table. It was then covered with a cardboard frame and moved into position in the center of the circle, facing along the zero line. Care was taken not to expose to light and spoil its dark adaption. The proper illumination having been set beforehand, the working light was turned out and the frame lifted. The animal's course could be followed by reference to the luminous points, and when the circumference was crossed, a flashlight was turned on and the reading taken of the angle turned. The isopod was then quickly covered with

the cardboard frame and returned to the center of the circle. Positions were alternated for each response: first the left side illuminated, then the right. Each animal was allowed to make about ten responses. The bulk of the work was done on *Oniscus*, of which only ten individuals were available. At the close of the experiment with each animal the chip was removed as described and the isopod returned to its bottle. Responses were measured in the actual number of degrees turned from the zero line in the direction headed; distributions were made of these and the means calculated.

RESULTS.

1. Responses in the Absence of Light (*Fig. 2, i*).—All species of isopods tried in darkness were found to move out of the circle in entirely a chance way, so that the statistical average of the paths, + and − from the zero line, is 0°, or very close. This shows that (*a*) there is no directive stimulus acting, and thus serves as a check on the apparatus and method; (*b*) that the stimuli initiating locomotion are not light stimuli; (*c*) that any internal or external stimuli setting up physicochemical processes which influence the path of the animal in any way act in a purely chance fashion. Chief among these stimuli is the stereotropism of the isopod, which causes it to remain in close contact with portions of the frame, and may be the cause of the undirected movements in the dark. Lifting the frame will almost invariably disturb the animal, and so furnish uncontrollable stimuli. Care had to be taken that the animal was headed with its axis coinciding with the zero line, as a very slight error here would give rise to a considerable angular deflection.

2. Responses to Bright Light (*Fig. 2, a*).—When subjected to rather high lateral illumination, the isopods respond by turning sharply away from the light and moving out of the circle in the negative region. The usual response is preceded by a period during which the animal remains motionless in the center of the circle—this is always definite in the normal response—and if no such pause of at least 2 seconds is made, the response is considered abnormal and discarded. Following this pause there is a period of antennary movement, followed closely by or begun immediately upon locomotion. The path with a very few exceptions is away from the light, and may be gradually curved,

or sharply broken. Usually the animal turns far enough until the curved carapace shades the illuminated eye, at which time the curving movements become less marked. Variations occur, as they do in the absence of light, but again they affect the response in a chance way. In bright light, however, the strong negative response tends to mask all these slight variations, and quickly eliminates any which tend to take the animal toward the light.

In bright light the response is maximal for a wide range of intensity with a given species. A comparison of several species was made, and the following was found. The average response of *Oniscus asellus* was 55° (± 2°) for illumination of 1.50 m.c.; of *Porcellio* (*Porcellio scaber* and *Porcellio rathkei* used indiscriminately), 31° (± 5°); of *Armadillidium*, 16° (± 9°—based on only twenty responses). In darkness *Oniscus* and *Porcellio* averaged 0° (± 3°, ± 5°, respectively). *Oniscus* is more negative and considerably more consistent in its responses, and so was used exclusively in the rest of the experiments.

The constancy of the maximal response which is elicited throughout a wide range of intensity is shown in Fig. 2, *a* to *c*, which are distributions of responses of ten individuals to three intensities of illumination ranging from 1.50 to 0.0026 m.c. For this range the action of the light is so strong as to mask other stimuli almost completely. The light rapidly turns the animal until the convex carapace shades the illuminated eye, and the active directive effect of the light is absent, although the turning movement may occasionally persist. This angle need not be 90°, but, due to the shape of the thorax, is such that when the animal has turned through an angle of about 50–60° both eyes are in the shade. Random movements occurring here might or might not bring the animal around so that one eye was again illuminated, and so bring about a reorientation—these certainly occur in a certain percentage of the cases. Because of this anatomical peculiarity, the strength of illumination required to elicit a maximal response need only be that which will produce enough photochemical product in the time of reaction to turn the animal until both eyes are in the shade in the radial distance of 10 cm.

3. Responses to Dim Light.—If the illumination is reduced sufficiently, it is clear that a critical point should be reached below which there

is not enough photochemical effect to turn the animal through the maximal angle in the radial distance of 10 cm. The distributions of

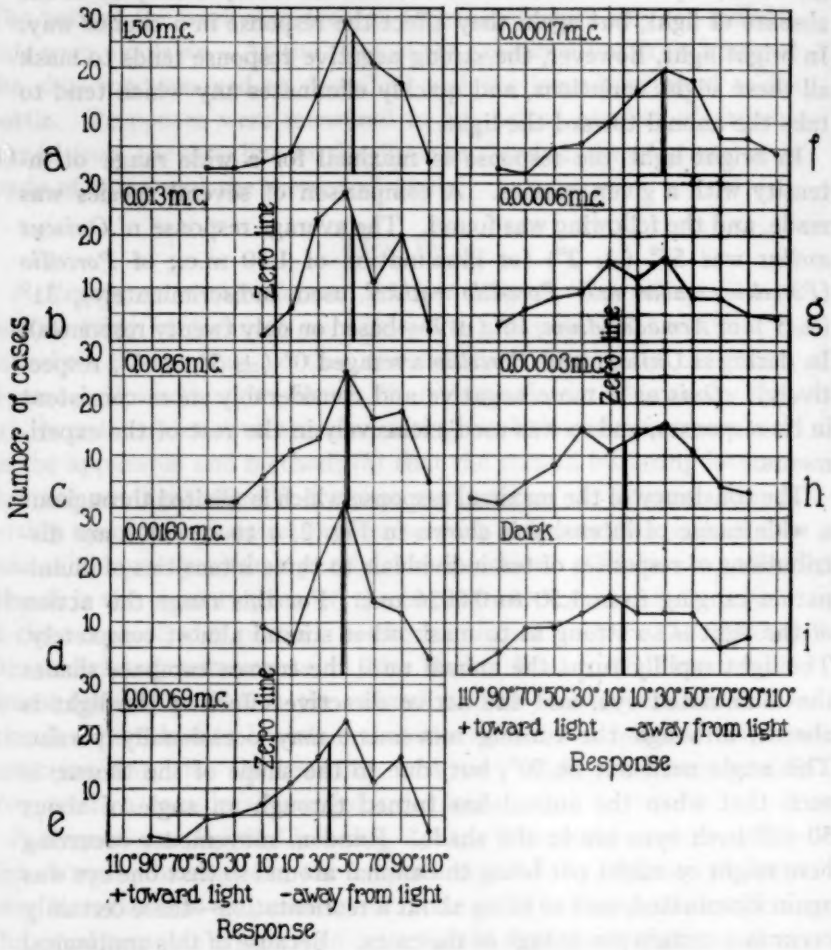

FIG. 2. Distributions of about 100 responses each of *Oniscus asellus* to light of various intensities. Responses measured as angular deflection of path from zero line, along which animals were headed. Heavy line is arithmetic mean of each distribution.

responses in illuminations below this value would be chance distributions, still skewed, with the mean at a point which would be deter-

mined by the photochemical effect of the given intensity of illumination. At these low intensities the deviations which arise from obscure causes will not be heavily overbalanced, and as a result care must be taken that they are not influenced in any regular way, such as failing to have the animal face along the zero line. The luminous material necessitated by these low intensities was used at the higher ones so that the results might be comparable.

The critical intensity was found to be somewhere in the region of 0.0026 m.c. At this intensity and above, the mean response is close to 55°. Below this value, as shown by Fig. 2, *d* to *h*, the distribution

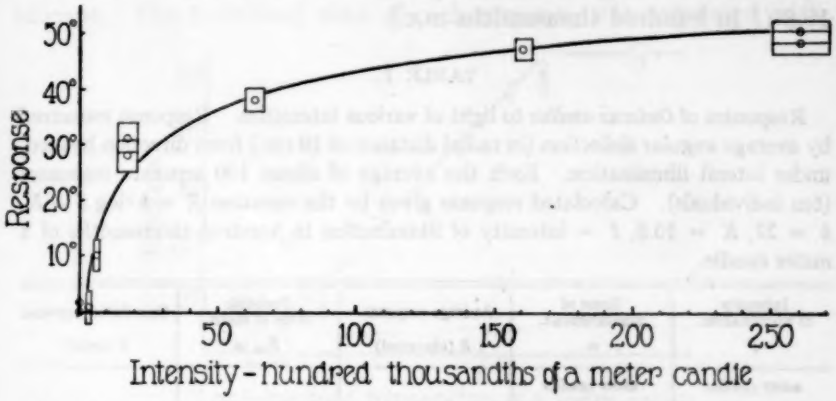

FIG. 3. Average response (angular deflection—means of Fig. 2) of *Oniscus asellus* to various intensities of light. Each point the average of about 100 separate responses. Rectangle surrounding each point indicates extent of probable error of both mean response and measurement of intensity.

flattens out and the mean approaches 0°. At the intensity below which the light produces no measurable results, the distribution is purely chance, like that of responses in darkness (Fig. 2, *i*). This threshold is about 0.00003 m.c.

Whether or not the mean responses at various intensities may be dealt with quantitatively is open to question. If the effective photochemical substance is formed in the period before locomotion starts, and its quantity little affected by changes of illumination (which would probably be rather slight due to the shortness of the period of turning and the marked convexity of the eye), then every degree

of turning would be an equal measure of the effectiveness of the light, whether it be a degree close to the zero line or not. A comparison is made on this assumption, using the mean response as a measure of the reaction of the animal.

If the average responses to the lower intensities are plotted against their respective intensities, the curve shown in Fig. 3 is obtained. The nature of this curve is indicated by Fig. 4, in which the average response is plotted against the logarithm of the respective intensity. Between the threshold and the critical value for maximal response the points lie fairly close to the line drawn through them. The equation of this line is $R = k \cdot \log I - K$, in which $k = 27$ and $K = 10.8$ (I in hundred thousandths m.c.).

TABLE I.

Responses of *Oniscus asellus* to light of various intensities. Response measured by average angular deflection (in radial distance of 10 cm.) from direction headed, under lateral illumination. Each the average of about 100 separate responses (ten individuals). Calculated response given by the equation $R = k \cdot \log I - K$, $k = 27$, $K = 10.8$, I = intensity of illumination in hundred thousandths of a meter candle.

Intensity of illumination. I	Error of measurement. ±	Average response. R (observed)	Probable error of mean. E_m ±	Calculated response. R (calc.)
meter candles	*meter candles*			
1.50	0.01	55.0° 52.4°*	2°	(55°)
0.013	0.001	54.4°	2°	(55°)
0.0026	0.0001	51.2° 48.6°*	2°	54.5°
0.00160	0.00003	47.3°	2°	48.6°
0.00069	0.00003	38.1°	2°	38.9°
0.00017	0.00003	30.4° 27.7°*	3°	22.4°
0.00006	0.00001	10.0°	3°	10.3°
0.00003	0.00001	1.1°*	3°	2.2°
Dark		1.9° 0.0°*	3°	(0°)

* Check experiments carried out under carefully controlled voltage of lamp. Time measurements mentioned in text (below) made for these.

Table I gives the average responses of *Oniscus* to the various intensities tried, and also the calculated values of the responses in the range between the threshold and the critical value for maximal response, on the basis of the above equation.

The photokinetic effect is probably quite small, and the factors concerned with the time of response are probably other than light. The actual time required for the entire response from the time of lifting the frame until the animal crossed the circumference was measured by a stop-watch for the entire range of intensity, including darkness. The average was found to vary independently of the intensity between 22.0 and 32.7 seconds—differences scarcely significant. The individual time of each response was correlated with

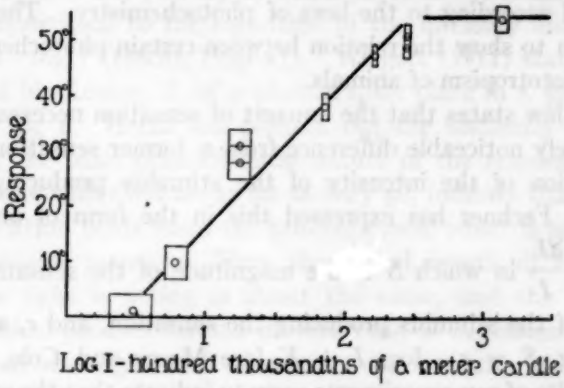

FIG. 4. Response of *Oniscus* plotted against logarithm of intensity.

the magnitude of turning, for each intensity, and the coefficient of correlation was found to vary between $+0.17$ and -0.10 (± 0.07), indicating clearly that there is no relation between time taken to make a response and amount of angular deflection.

DISCUSSION.

The constant maximal response which these forms make to light above a certain critical intensity can be accounted for on purely anatomical grounds, being determined by the convexity of the carapace and the position of the eyes. The specific differences among the isopods can be explained on these grounds, *Porcellio* being more variable

and having a slightly more convex carapace, *Armadillidium* having a considerably more convex thorax.

Below this critical intensity the responses fall off regularly with decrease in intensity. Variations which are caused by obscure processes due to uncontrollable stimuli—"random movements"—are here no longer masked by the light reaction, but are still affected so that the distribution is skewed. The mean response will then be determined by the light, in accordance with the muscle tonus theory of Loeb. According to this theory, the amount of turning is determined by the difference in tonus of body muscles on opposite sides of the body, and this difference is in turn due to a difference in the amount of photochemical product in the two receptors. This amount is determined according to the laws of photochemistry. These experiments seem to show the relation between certain photochemical laws and the phototropism of animals.

Weber's law states that the amount of sensation necessary to produce a barely noticeable difference from a former sensation is a constant fraction of the intensity of the stimulus producing the first sensation. Fechner has expressed this in the form of an equation $dS = c \cdot \frac{dI}{I}$, in which S is the magnitude of the sensation, I, the intensity of the stimulus producing the sensation, and c, a constant. Integrating, $S = c \cdot \log_e I + K$ (see Moore and Cole, 1920–21).

The results of my experiments seem to indicate that the phototropic responses studied follow at least this form. This is not at all singular, since Moore and Cole (1920–21) found that the beetle, *Popillia*, follows this relation, and Cole (1922–23) found that the ratio of circle diameters of the movements of asymmetric *Limulus* is proportional to the logarithm of the intensity of illumination—the higher the intensity the smaller the circles. Moreover, this same kind of a response takes the logarithmic form in the whip-tail scorpion (Patten, 1917). I have replotted Patten's data, putting response (angular deflection) against logarithn of intensity, and find that the points lie roughly on a straight line, indicating that *Mastigoproctus* also follows this form.

The similarity between Cole's results for *Limulus* (1922–23) and mine for *Oniscus* indicates that the amount of turning in *Oniscus* is a

measure of exactly the same thing as the circle diameters of *Limulus* and that the negative response is in reality an arc of a limited circus movement.

Hecht (1919–20), has pointed out that a logarithmic relation may be merely a coincidence with the Weber-Fechner law, and that the real basis is probably a photochemical one. He has found the reactions of *Mya* to be definitely of a photochemical nature, and explains them on the basis of the reaction $S \underset{\text{dark}}{\overset{\text{light}}{\rightleftharpoons}} P+A$, in which S is the amount of photosensitive substance present, and P is the photochemical product with its accessory A. The photochemical effect is produced by P and A (through a simple reaction which they catalyze), and is proportional to the logarithm of the intensity and the time, $E = k \cdot t \cdot \log I$ (Hecht, 1920–21). Weigert (1911) states that the amount of blackening, S, of a photographic plate in a given time is $S = k \cdot \log I$. Hecht has pointed out the similarity of the two, indicating the basic photochemical nature of the responses of *Mya*.

My results with *Oniscus*, as far as they go, indicate that this same general relation holds when the photochemical effect is measured by the phototropic response. Here the actual length of time during which the light is acting is about the same, and the amount of photochemical product formed in this time is dependent upon the intensity of the light and determines the amount of turning, in accordance with Loeb's theory.

Hecht (1922–23) has pointed out that the amount of freshly formed photochemical product in excess of the amount already present in sensory adaptation is what determines the muscular response, rather than a ratio between the two. This is also found to apply to a tropic response. The amount of product present in the shaded eye of *Oniscus* (if completely dark adapted) would be zero. The amount in the illuminated eye is the photochemical effect, E, and is determined according to the equation $E = k \cdot t \cdot \log I$ (t is constant). The ratio $E : 0$ is indeterminate, but the *excess* of the amount in the illuminated eye over that in the shaded eye is E, and is determined by the above equation, as indicated by results with *Limulus*, *Mastigoproctus*, and *Oniscus*. This is further borne out by the work of Patten (1915) on blowfly larva. The orientation of these animals subjected

to opposed beams of light was found to be dependent only upon the ratio of the intensities on the two sides. The photochemical treatment of these data is significant. Let I_1 be the intensity of the light shining on one side, and I_2 the intensity on the other. Then the photochemical effects in the two receptors will, according to the above equation, be

$$E_1 = k \cdot t \cdot \log I_1$$

and

$$E_2 = k \cdot t \cdot \log I_2$$

in which E_1 and E_2 are respective photochemical effects. If the tendency to turn (R) is determined by the difference in photochemical effect in the two receptors, this will be

$$R = E_1 - E_2 = k \cdot t \cdot (\log I_1 - \log I_2),$$

or

$$R = k \cdot t \cdot \log \frac{I_1}{I_2}$$

This means that the photochemical effect is proportional to the logarithm of the *ratio* of the two intensities, regardless of their absolute values. The tendency to turn on account of the resulting muscle tonus will therefore be the same as long as the ratio of the intensities is the same. As the animal turns, the effective intensity—which is the intensity of the beam at that point times the cosine of the angle made by the norm of the surface and the ray of light—is changed until it is equal on both sides, and $I_1 = I_2$, whence $\frac{I_1}{I_2} = 1$, and $\log \frac{I_1}{I_2} = 0$, and so $E_1 - E_2 = 0$, and the animal is oriented.

Similar reasoning could be applied to the experiments of Northrop and Loeb (1922–23) on *Limulus*, if the orientation were to result regardless of the absolute value of the intensity, since in this material the validity of the equation $E = k \cdot \log I$ has been proven by Cole (1922–23).[1]

[1] Rough experiments performed by H. S. Mayerson and myself at Woods Hole, Massachusetts corroborated Northrop and Loeb's findings, and indicated that the orientation is actually determined by the ratio of the lights, regardless of their absolute intensities.

These results and considerations throw some light on the photochemical basis of phototropism, since they link up Hecht's work on *Mya* with a phototropic response. This can best be understood on the assumption that muscular contraction determining the tropism is proportional to the amount of photochemical product formed in the illuminated photoreceptor, which in turn is determined by the intensity of illumination on the receptor in accordance with laws governing a photochemical process such as the reduction of silver salt on a photographic plate. There can, therefore, be hardly any other conclusion but that, as Loeb originally pointed out, phototropism is the result of a difference in tonic contraction of opposing muscles determined in a purely photochemical way by the excess of decomposed photosensitive substance in one photoreceptor.

SUMMARY.

1. The negative phototropism of certain land isopods was investigated over a large range of intensities, especially low ones. The responses were determined quantitatively by measuring the angle through which an animal turned away from a line perpendicular to the rays of light.

2. In the absence of light the undirected movements set up by obscure stimuli were such as to compensate each other statistically, the average path being a movement in the direction in which the animal was headed.

3. Over a large range of intensities (0.0026 m.c. up) the average turning is maximal, about 55° (*Oniscus*). This maximal response is due to an anatomical peculiarity, in that the carapace cuts off the light on the eye after the animal has turned 50–60°. This peculiarity probably accounts for specific differences among land isopods. Any light, therefore, which is strong enough to turn an animal through this maximal angle in a radial distance of 10 cm. will give results whose mean will be maximal.

4. Below 0.0026 m.c. the amount of angular deflection becomes less and less, in proportion to the logarithm of the intensity, until at 0.00003 m.c. the movements are the same as in darkness.

5. This proportionality between amount of turning and the logarithm of the intensity indicates the photochemical nature of phototropism

on the basis of Hecht's work with *Mya*. As a result, Loeb's theory of phototropism may then be stated in the mathematical form

$$R = E_1 - E_2 = \log \frac{I_1}{I_2}$$

in which I_1 and I_2 are the two intensities, E_1 and E_2, their respective effects, and R, the muscular action set up by the difference in photochemical effect on the two sides.

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OBSERVATIONS ON THE TRANSPORT OF CARBON DIOXIDE IN THE BLOOD OF SOME MARINE INVERTEBRATES.

By THOMAS R. PARSONS and WINIFRED PARSONS.

(*From the Zoological Station, Naples, Italy.*)

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While the respiratory properties of the blood of vertebrates, and more particularly of mammals, have formed the subject of numerous investigations, the study of the corresponding functions in invertebrates has received but little attention. Apart from the writings of those authors who have considered the question of the combination of oxygen with the blue copper-containing pigment, hemocyanine, the most important article dealing with the gas content of the circulatory fluids of invertebrates is that of Winterstein (1). This contains an account of the actual quantities of oxygen and of carbon dioxide contained in the bloods, drawn directly from the vessels of a number of marine animals, and serves to correct the erroneous figures previously given by Griffiths (2) who had claimed to have obtained values of the same order of magnitude as those which are normal for vertebrates. By means of the blood gas pump Winterstein showed that the circulating fluids of these invertebrates contained much smaller quantities of gas than Griffiths has supposed. The main result of this investigation was, therefore, to fix the physiological limits of gas content in the bloods of the forms examined. Since Winterstein's work the oxygen and carbon dioxide dissociation curves for mammalian, and more particularly for human, blood have been completely worked out (3). The dissociation of oxygen in invertebrate blood has also received some attention, more particularly in those forms in which either hemocyanine or hemoglobin is present; but the relationships of the carbon dioxide in these forms seems to have been almost entirely neglected. Recently, however, investigations bearing upon this subject have been carried out by Collip (4) whose paper became accessible to us on our return from Italy. Collip used the Van Slyke apparatus

(5), and with it determined the total content of combined carbon dioxide in the body fluids of a large number of marine forms after these fluids had been equilibrated with atmospheric air. In a few cases, also, the carbon dioxide content of the fluids was measured after equilibration with alveolar air. From Collip's results it is seen that, in these cases, the increased amount of carbon dioxide taken up at a pressure of CO_2 of about 40 mm. Hg over and above that taken up at the low tension of the gas existing in the atmospheric air is such as would be accounted for by the extra volume of the gas taken up in physical solution at the higher tension. This means that in the bloods of all the forms examined practically all the available alkali was combined with carbon dioxide at so low a tension of carbon dioxide as that existing in ordinary atmospheric air. It is evident, therefore, that the properties of these fluids are very different from those of human blood, in which, at body temperature, the amount of carbon dioxide taken up increases gradually as the tension of the gas is increased, so that it is not until comparatively high tensions are reached that the greater part of the available alkali has been converted to bicarbonate.

Thus the chief object of Winterstein's work was to measure the gas content of the body fluids of invertebrates, while Collip's investigations were directed towards the determination of the alkali reserve, or total concentration of available alkali in these fluids. Our object, however, has been to plot out the complete carbon dioxide dissociation curves of the body fluids of the commoner and larger invertebrates found in the Mediterranean at Naples, and to look for such correlation as might obtain between the form of curve given by any species and its particular respiratory conditions and habits.

Methods.

Naturally the methods used for withdrawing the blood or other body fluid varied according to the particular animal experimented upon. We shall therefore describe them separately along with the results obtained for each species. Suffice it to say that portions of the fluid obtained were saturated at a temperature of 15°C. with analysed mixtures of air and carbon dioxide by means of the apparatus already described for use with human blood (6). A measured volume of fluid was then run below the surface of 2 cc. of N/40 baryta solution

contained in one bottle of a Barcroft differential apparatus, in which the CO_2 was liberated by acidification in the usual way. The apparatus had previously been calibrated by the use of a standard solution of sodium carbonate. In each case we not only plotted the carbon dioxide dissociation curve of the blood but also attempted to fix the physiological limits by analysing the fluid collected without loss of gas directly from the body into a graduated pipette. In a number of cases we used the Van Slyke apparatus (5) for the determination of the carbon dioxide contents of the fluids, extracting the whole of the gas liberated on acidification and exposure to a vacuum, and afterwards determining the amount of carbon dioxide in this gas by exposure to potash solution contained in a tiny absorption pipette attached to the side tube of the apparatus. We shall now describe the results of the observations we made on the bloods of the various forms we examined.

I.

Maia squinado.

The blood was collected by cutting through the appendages with stout scissors and collecting the fluid as it dripped from the cut surface. The animal showed a great tendency to throw off the injured limb by a process of autotomy at its base. If this happened before sufficient blood had been collected, further limbs were operated upon. The blood so collected has a reddish orange color owing to the presence of the pigment hemerythrin; hemocyanine is also present, but its blue color is masked. This blood does not clot, the animal relying upon its power of autotomy to prevent bleeding to death. The white corpuscles were caused to settle by gentle centrifuging, and the blood was then preserved in the ice chest until it was examined on the 2 days immediately succeeding that on which it was drawn. The points we obtained on the carbon dioxide dissociation curve for this blood were as follows:

CO_2 tension, *mm. Hg*	3.4	6.2	12.0	23.2	43.5	71.0	108.1
CO_2 content per 100 cc., *cc.*	11.9	15.3	17.5	18.6	23.4	26.9	29.6

These values are plotted in the figure, from which it will be seen that the blood of *Maia* possesses a carbon dioxide dissociation curve at sea

temperatures which, over the greater part of its range, ascends much less rapidly than does the corresponding curve for oxygenated human blood at body temperature which is reproduced from Christiansen, Douglas, and Haldane's paper (3) for the sake of comparison. In fact, the curve for *Maia* blood is practically parallel to that showing the amount of carbon dioxide dissolved in pure water at 15°C. But it will be seen that the blood of this animal contains a considerable amount of combined carbon dioxide and that the dissociation curve rises quite steeply at the lowest tensions of this gas. It was of interest, therefore, to determine the tension or content of the blood as it circulates in the animal in order to discover which portion of the curve —the initial steep portion or the later more gently ascending portion— falls within the physiological limits and so is used by the animal. In order to do this we collected a sample of the animal's blood directly into a 1 cc. measuring pipette attached to a hypodermic needle the point of which was inserted into a leg stump. The fluid flowed easily into the pipette from which a measured volume was run off and analysed at once. The carbon dioxide content of this sample was found to be 9.7 cc. per 100 cc. of blood. On another occasion we analysed the blood passing from the gills into the so called pericardium by inserting the needle into a small hole drilled in the dorsal carapace of the animal, in the distinctly marked area which marks the position of the heart below. During the withdrawal of the blood the animal was kept in a small tank of sea water with the back just projecting. The carbon dioxide content of this fluid was 4.9 cc. per 100 cc. of blood. After this experiment, which was carried out by means of the differential apparatus, a second sample of blood was withdrawn in a similar manner and analysed by means of the Van Slyke apparatus. A carbon dioxide content of 12.3 volumes per cent was now observed. From this result it seemed likely that carbon dioxide was accumulating in the animal's body in consequence of its confinement to a comparatively small volume of water. The specimen was therefore left untouched until the evening when further samples of its blood were withdrawn through the hole previously made in the carapace. One of these samples analysed in the differential apparatus was found to possess a carbon dioxide content of 20.4 cc. per 100 cc.; the second sample in the Van Slyke apparatus gave a corresponding value of 18.1.

By the time these last samples were taken the animal had become very "dull" and showed but little response when disturbed. Collip (4) mentions the need for keeping the respiratory conditions of an animal as normal as possible during determinations of its blood gases, and Winterstein (1) has followed the gradual accumulation of carbon dioxide in the pericardial blood of *Maia* while the animal was kept in moist air. But Winterstein's figures for the carbon dioxide content of the bloods of specimens of *Maia* kept under normal respiratory conditions are much higher than those which we observed.

II.

Palinurus vulgaris.

The blood of this lobster, obtained by cutting the appendages, presents a marked contrast to that of the crab *Maia*. In the first place the blue color of the hemocyanine is here not masked by the presence of other pigments. And secondly, the blood on standing clots to a firm jelly. This clotting can be prevented by the addition of potassium oxalate, but only in such amounts as would be likely to influence the carbon dioxide-combining power. These circumstances would seem at first sight to present an insuperable difficulty in the examination of this fluid, but we found it possible to "defibrinate" the blood by allowing complete clotting to occur and grinding the resulting jelly with a little coarsely powdered glass in a small mortar. In this way the threads of fibrin are broken and the original firm clot is converted almost entirely into liquid, the amount of solid remaining being surprisingly small. The glass was filtered off through a layer of clean linen at the pump, and the resulting dark blue defibrinated blood was used for the following experiments.

CO_2 tension, *mm. Hg*.....	4.0	5.8	7.4	9.2	19.3	20.7	46.1	119.1
CO_2 content per 100 cc., *cc.*.....	11.2	11.3	11.4	14.4	19.8	23.1	31.4	49.2

From the figure it will be seen that the carbon dioxide dissociation curve in this form rises more gently and to a greater maximum value for the combined carbon dioxide than does that obtained for the blood of *Maia*. The blood of *Palinurus* behaves as if its bicarbonate equilibrium is more profoundly modified by the presence of feebly acid

substances than is that of the blood of any other of the forms we examined.

Owing to the rapid clotting of this blood it was difficult to ascertain the carbon dioxide content of the blood of *Palinurus* drawn straight from the body. By rapid working it was found to be possible to collect a specimen from the cut surface of a leg stump and to measure it off and dilute it in the Van Slyke apparatus before it solidified. In this way we obtained a carbon dioxide content of 5.4 cc. per cent—a value which shows that in the case of this animal, as in *Maia*, it is the initial steep portion of the dissociation curve which is in use under physiological conditions.

III.

Octopus vulgaris and Octopus macropus.

The blood of the *Octopus* was collected by employing the technique devised by Fredericq (7). The animal was nailed by its tentacles to a board and then covered by a duster which left the body exposed. The respiration of the animal was maintained by inserting a glass tube conveying aerated sea water into the mantle cavity. An incision was then made into the body wall in the mid-dorsal line in order to expose the main dorsal artery. This was suitably ligatured to permit of the insertion of a long glass cannula, through which the blood was readily collected in abundance. An alternative method for dealing with this somewhat vigorous and troublesome, but interesting animal is to enclose its tentacles in a suitable bag and to support its body in a wire stand of appropriate shape during the operation. The blood so obtained possesses a deep blue color due to the presence of hemocyanine. It does not clot, the only change observed on standing being an agglutination of the leucocytes.

Our first experiments were carried out on the blood of *Octopus vulgaris* with the following results.

CO_2 tension, *mm. Hg*	2.4	4.5	7.8	14.6	26.2	52.8	102.0	148.7
CO_2 content per 100 cc., *cc.*	5.6	8.4	9.1	11.4	14.5	19.9	26.6	29.5

These figures show a carbon dioxide-combining power less than that observed in the case of *Maia*, but as Dr. Quagliariello, who kindly estimated for us refractometrically the percentage of hemocyanine

in the sample of blood, reported the abnormally small value of 2.9 per cent (about one-third of the amount usually found in this animal), it seemed possible that the specimen we had used was in some way abnormal or that the blood during its collection had become inadvertently mixed with sea water. The results were, therefore, not plotted in the figure. The curve labelled *Octopus* is plotted from the following results, obtained with the blood of a specimen of *Octopus macropus*.

CO_2 tension, *mm. Hg*	6.7	26.3	44.5	80.9	113.9
CO_2 content per 100 cc., *cc.*	12.2	20.3	28.4	34.3	41.0

It will be seen that the carbon dioxide-combining power of this blood is greater than that of *Maia*, and further that the curve has a more gentle slope. A determination of the carbon dioxide content of the arterial blood drawn directly into a pipette from the vessels of another specimen of *Octopus vulgaris* gave a value of 3 cc. CO_2 per 100 cc. of blood. Winterstein found values ranging from 3.94 to 7.09 cc. CO_2 per cent for the arterial blood and from 5.62 to 7.83 cc. CO_2 per cent for the venous blood of *Octopus vulgaris*, so that the physiological limits in this animal also include only the initial steep portion of the curve.

We carried out a few further observations on the respiratory phenomena of this animal. For example, we made a series of determinations of the difference in carbon dioxide content produced as the water circulated through a mantle cavity. In order to collect the outgoing water a short length of glass tube of appropriate diameter (about 1 cm.) was tied lightly into the funnel of the animal, and the collecting and measuring pipette was inserted into this. Each portion analysed consisted of a mixture of several separate fractions of 1 cc. collected from each of a number of "expirations." Successive analyses with the differential apparatus gave values of:

4.6, 5.1, 3.2, 3.1, 4.4, mean 4.1 cc. CO_2 per cent.

The "inspired" water gave readings of:

3.9, 4.4, 3.3, mean 3.8 cc. CO_2 per cent.

Even these few observations show how efficient is the washing out of the respiratory cavity by the sea water, and how large must

be the amount of water passing over the gills in comparison with the amount of carbon dioxide which has to be removed.

We also made an attempt to discover whether the carbon dioxide-combining power of the blood of *Octopus* is influenced by the oxygen content in any way similar to that well known to exist in mammals.

The following three points were obtained by saturating the blood with mixtures of nitrogen and carbon dioxide.

CO_2 tension, *mm. Hg*	45.5	104.1	122.1
CO_2 content per 100 cc., *cc.*	27.1	35.2	38.1

They are represented as black triangles in the figure, from which it will be seen that they fall somewhat below the curve for the oxygenated blood. These measurements are too few in number to enable us to draw certain conclusions, but it appears that oxygenation of hemocyanine produces no change in the carbon dioxide-carrying power of this blood comparable to the important change produced by the oxygenation of the hemoglobin in the blood of a mammal.

It should be mentioned that in these determinations the mixture of blood and baryta solution in the bottle of the differential apparatus was shaken in air before the acid was added to expel the carbon dioxide, in order to saturate the hemocyanine with oxygen and so to ensure that the readings were made under conditions similar to those which obtained during the determination of the "oxygenated" points.

IV.

Phallusia (ascidia) mammillata.

The blood of the ascidians is particularly interesting in some particulars. For Henze (8) has shown that while its plasma is faintly alkaline and possesses a salt content not very different from that of sea water the blood corpuscles contain a fluid which is strongly acid owing to the presence of about 3 per cent of free sulphuric acid. Furthermore, certain of the corpuscles carry a complex vanadium-containing chromogen which is thought to act as a catalyst during oxidative processes. The determinations by Quagliariello (9) of the reaction of the blood of these animals gave values decidedly less

alkaline than those given by other invertebrate bloods. We collected blood from these animals by making a careful incision through the basal part of the mantle on the side opposite to the expiratory orifice so as to expose the thin walled "heart." On puncturing this vessel by means of a hollow needle the greenish blood was quickly expelled. The color is due to the corpuscles. These were found to settle very rapidly on centrifuging, leaving a clear colorless plasma above a thin layer of cells. We have two determinations on the plasma obtained in this way from one animal and two on the whole blood of another specimen.

	Blood.		*Plasma.*	
CO_2 tension, *mm. Hg*	27.4	51.4	27.1	52.7
CO_2 content per 100 cc., *cc.*	0.3	2.9	2.9	5.3

From the figure it will be seen that the carbon dioxide-combining power of the plasma is appreciably less than that of sea water, while that of the whole blood—probably on account of the presence of the acid corpuscles—is still smaller. Winterstein similarly found a vanishingly small quantity of carbon dioxide in ascidian blood which had been equilibrated with air.

V.

Aplysia limacina.

The last body fluid for which we have data is the cœlomic fluid of this gastropod. The clear limpid liquid is obtained in abundance on making a cut in the foot of the animal. It is completely devoid of pigments and from the following results it will be seen that its carbon dioxide dissociation curve is practically identical with that of sea water.

CO_2 tension, *mm. Hg*	0.9	1.2	4.5	7.0	11.5	27.1	57.3	83.6	107.7
CO_2 content per 100 cc., *cc.*	5.0	5.4	5.5	5.8	7.5	8.3	11.4	10.7 (?)	14.5

A determination of the total CO_2 content of a sample of the fluid allowed to flow directly into a pipette attached to a hollow needle inserted into the foot of the mollusc gave a value of 4.4 cc. total CO_2 per 100 cc. of fluid—a value not appreciably different from the 3.7

cc. per cent which we found to be present in the sea water circulating in the aquarium tanks. It is to be concluded therefore that the body fluid in this form has no special function in connection with the carriage of carbon dioxide.

VI.

Sea Water.

We have three measurements on the sea water of the Gulf of Naples as follows:

CO_2 tension, *mm. Hg*	0.8	58.1	125.3
CO_2 content per 100 cc., *cc.*	4.7	10.9	18.3

These are included in the figure and indicate a distinct combining power for carbon dioxide. As just mentioned, the sea water circulating in the aquarium tanks had a total content of CO_2 of 3.7 cc. per 100.

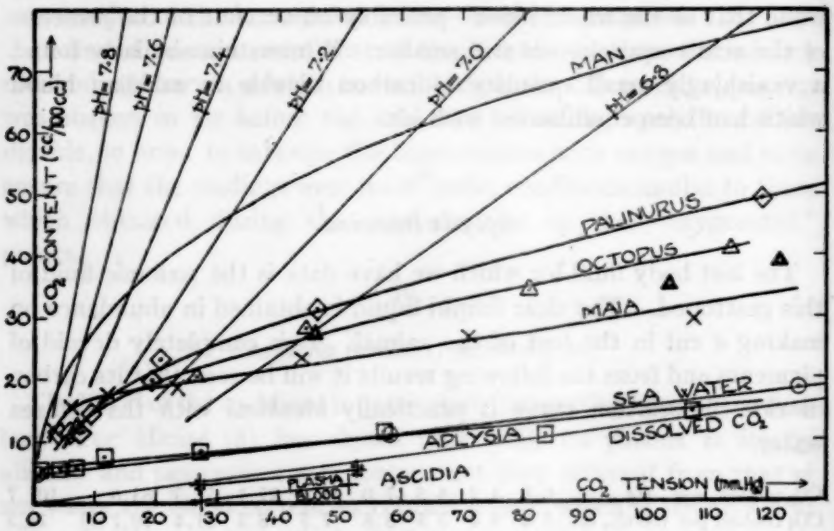

FIG. 1.

GENERAL CONCLUSIONS.

Although the results we have recorded merely serve to indicate the possibilities of this interesting field of investigation, we have sufficient data to enable us to draw certain general conclusions. In the first

place it is evident that the bloods of the more highly developed marine invertebrates, such as the active *Crustacia* and the *Cephalopods*, are specially adapted for the carriage of carbon dioxide. The quantity of carbon dioxide taken up by the blood of *Maia*, *Palinurus*, or *Octopus* at any given tension of the gas is, in general, about twice or three times as great as that which is taken up by sea water under the same conditions. On the other hand, the blood of a slow, creeping form, such as *Aplysia*, or of a sessile animal such as the ascidian *Phallusia* shows no more adaptation for the carriage of carbon dioxide than does sea water.

But our estimations of the CO_2 content of the blood as it circulates in the bodies of these more active invertebrates show that the conditions of transport of this gas differ considerably in some respects from those which obtain in mammals. For the invertebrate blood in the body contains only a relatively small quantity of carbon dioxide, averaging in the forms we examined from 3 to 10 cc. per 100 cc. of blood. This forms a marked contrast with the condition found in mammals where even the arterial blood contains about 50 cc. of CO_2 per 100 cc. of blood. The invertebrate, therefore, works at a very low CO_2 tension. There is a twofold significance in this circumstance. In the first place, it means that only the first portion of the carbon dioxide dissociation curve is in use in the respiratory mechanism. Now an inspection of our curves will show that at these low carbon dioxide tensions the dissociation curves tend to be steeper than at higher tensions. As we intend to show in a later paper it can be proved mathematically that, other things being equal, a blood with a carbon dissociation curve of moderate steepness, *i.e.* one in which the carbon dioxide content of the blood increases fairly rapidly with increase of carbon dioxide tension, is a more efficient carrier of the gas from the tissues to a respiratory surface than a blood in which the dissociation curve is either steeper or flatter. It would seem as if the active invertebrates avoid the use of too flat a part of their CO_2 dissociation curves by working over the initial steeper portion.

Furthermore, it is seen that over the range of this initial steep portion of the curves the changes of reaction produced by the uptake of carbon dioxide are much smaller than at higher tensions of the gas; for these initial portions of the curves are more nearly parallel to the

lines of constant reaction calculated for a temperature of 15°C. according to Hasselbalch's method (10) on the assumption that the whole of the combined CO_2 is in the form of sodium bicarbonate. It is evident also that on this assumption the hydrogen ion concentration of the blood of invertebrates (with the exception of the tunicates) would appear to be practically the same as that of the warm-blooded vertebrates—a conclusion confirmed by the direct measurements of Quagliariello (9). On the other hand, our measurements do not lend support to the idea put forward by Collip (4) that in order to maintain an appropriate faintly alkaline reaction an invertebrate needs to retain carbon dioxide in its blood at a comparatively high tension. This idea was based on the observation that at comparatively high CO_2 tensions the blood of invertebrates contains considerably more sodium bicarbonate than does sea water. But our curves show that this is no longer true at the lower values of carbon dioxide tension, the amount of sodium bicarbonate falling off more rapidly in the blood than in the sea water with diminution of the carbon dioxide tension so that in order to maintain an appropriate reaction in the blood only a comparatively small tension of CO_2 is required. The largest amount of carbon dioxide that we found present in the circulating blood of any of the types examined was 9.7 cc. per 100 cc. of blood in the case of *Maia*, and in most cases the amount was considerably less. But even this lowest value corresponds to a tension of CO_2 of only about 3 mm., so that the tension gradient across the gill membrane must be even less than this. We would emphasize rather the circumstances that as the portion of the dissociation curve over which the reaction is approximately constant is of but small extent, it is necessary that in an active form like *Octopus* the carbon dioxide produced should be removed rapidly lest an accumulation of it should cause the limits of normal reaction to be exceeded; and this need is correlated with the extreme efficiency of the respiratory apparatus in this animal. It is interesting to notice that the mammal which, in order to obtain an appropriate reaction in the blood, has to work at relatively high carbon dioxide tensions where the dissociation curve is comparatively flat, secures a steeper physiological CO_2 dissociation curve in the body, and with it a more efficient carriage of carbon dioxide and a more constant reaction in the circulating fluid, in virtue of the effect of oxygenation on the carbon dioxide-combining power of its blood (3, 6).

Returning now to the consideration of the actual form of the dissociation curves we have obtained—it is a significant fact that it is in those forms such as *Maia*, *Palinurus*, and *Octopus* whose bloods are rich in proteins—particularly hemocyanine—that the initial steep portion of the curve is observed. This suggests that in these forms the blood proteins act as weak acids and expel carbon dioxide from the blood at the low tensions which include the physiological range, just as in vertebrates the hemoglobin similarly displaces carbonic acid from its combination with alkali metal. On the other hand the cœlomic fluid of *Aplysia* contains no pigment and only 0.00672 per cent of protein nitrogen (Bottazzi (11)) and shows no initial rapidly ascending portion of the CO_2 dissociation curve. This is supported by the observation of Quagliariello (9) that the acid-neutralising power of the blood of an invertebrate is roughly proportional to its protein content. It seems as if the proteins of invertebrate blood like the blood proteins of vertebrates, exist in the form of sodium salts which are capable of giving up sodium for the transport of carbon dioxide as sodium bicarbonate. That this is so in the case of hemocyanine follows from the fact that the isoelectric point of this pigment occurs at a hydrogen ion concentration of 2.12×10^{-5} N, *i.e.* at a pH of 4.67 (Quagliariello (12)) so that in the alkaline blood of the invertebrates possessing it, hemocyanine will act as a weak acid. It is probable that the initial steep portion of the carbon dioxide dissociation curves which we have found to be of such importance in the respiration physiology of *Octopus*, *Palinurus*, and *Maia* is produced by the competition of this acid with carbonic acid for the available sodium of the blood.

In conclusion we desire to express our thanks to the Master and Fellows of Sidney Sussex College, Cambridge, for a generous grant towards the expenses of these investigations; to the Director and Staff of the Zoological Station at Naples for the wholehearted manner in which they afforded us the facilities we required; and to the Ambassadors of France and Italy who, on the recommendation of the Royal Society, were good enough to supply us with permits to take our packages of glass apparatus unopened through the customs houses of their respective countries.

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EXPERIMENTS ON TOLERATION OF TEMPERATURE BY DROSOPHILA.

By HAROLD H. PLOUGH and MAURICE B. STRAUSS.

(From the Biological Laboratory of Amherst College, Amherst.)

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In connection with experiments by one of us on the effect of high temperature on the amount of crossing over in *Drosophila* it became obvious that differences exist among various cultures of this fly in the ability to withstand temperatures above the normal range and in the ability to breed at these temperatures. A series of comparative tests was therefore undertaken in order to gain more accurate information along these lines.

Experiments on a single culture of *Drosophila melanogaster*, grown on sterile yeast medium, were reported by Northrop[1] in 1920. He wished to find out whether the offspring of cultures kept at high temperatures acquired the ability to tolerate temperatures still higher. He found that no such adaptation occurred, and that flies could be carried at temperatures above 29°C. for one generation only. These flies would however breed normally according to his findings if returned to normal temperatures for a period of 24 hours.

Since Northrop's work was upon a single stock of *Drosophila* only, it seemed of interest to us to determine whether there was not a difference in the upper temperature limit among different cultures of *Drosophila*, which might be genetically determined.

Our work indicates that with optimum food conditions it is only the exceptional stock of *Drosophila melanogaster* which will not breed at 31°C. Furthermore our work suggests the basis for the failure to breed at high temperatures as observed by Northrop and by ourselves.

[1] Northrop, J. H., *J. Gen. Physiol.*, 1919–20, ii, 313.

Our tests were carried on in electrically controlled bacteriological incubators. While by no means as constant as the apparatus used by Northrop, these were sufficiently so for all practical purposes. Checks were run on certain of the experiments in a water bath used in the Department of Chemistry which varied less than 0.1°C., with identical results in each case. The control incubator was carried at approximately 24°C. Most of the high temperature tests were made at 31°C. Approximate records of the limits of variation of these incubators were kept with Taylor maximum and minimum thermometers, and these showed variations of less than 1° on either side. In the case of the high temperature tests, Northrop[1] and Plough[2] have indicated that 29°C. was the critical temperature, which our experiment confirms, and our present material was constantly above this point in the high temperature work.

The medium used for the cultures was the usual banana agar, made up in fairly large quantities, placed in bottles, and kept in a refrigerator until used. A pinch of powdered magic yeast was dropped in each bottle before the paper and the flies were introduced. This medium, if carefully made and used within 2 or 3 days, gives far larger numbers of flies than the sterile solution of Northrop. The cultures are practically uniform and the conditions closer to those in the wild state.

EXPERIMENTAL.

Our first tests were made with a number of different cultures of *Drosophila melanogaster* collected from various widely separated regions. Following this series we tested a number of mutant stocks and wild stocks of several other species of *Drosophila*. Table I gives the results of these tests and also the source of the stocks tested. In general these tests were made with two bottles of each stock containing about three pairs of flies apiece. Whenever a test culture failed to develop, however, it was repeated at least once, so that each negative result represents at least four bottles tried.

Geographically Different Races of Drosophila melanogaster.—The data given in the table show in general that wild stocks of *Drosophila*

[2] Plough, H. H., *J. Exp. Zool.*, 1917–18, xxiv, 147.

melanogaster can be bred for indefinite periods at a temperature of 31°C. on banana agar. The stocks from Amherst, Georgia, and Sweden were carried for eight, ten, and eleven generations before they were lost and it is very probable that more careful methods, with a larger number of separate cultures would make it possible to carry them indefinitely. At 31°C. the flies begin to hatch within 8 days. They are smaller than under optimum conditions and somewhat less viable. All of these conditions appear to be due to the hastening of development with the result that the larvæ take in less food. Up to 31°C. there is no evidence that the stocks cited show any effects which make breeding beyond the second generation impossible as Northrop states. In the case of the Devil's Lake stock no difficulty was experienced in carrying the flies for fourteen generations and the stock was only discontinued at that point because we moved to Woods Hole where incubators were not available.

An interesting point in connection with these stocks has to do with the geographical origins. It was thought possible that stocks from southern or tropical climates might show greater resistance to heat than those from farther north. That this idea has no basis in fact is obvious. Stocks from Sweden and North Dakota go as well at 31°C. as those from Georgia and Florida.

In the case of the stock collected at Attleboro, the results follow those found by Northrop with his stock. Three distinct trials failed to give any offspring beyond the first generation. In addition the stock has been tested at least as many times more in another series of tests with identical results. This stock is as viable as any of the others at normal temperatures. It differs only in that it has been kept in culture since the summer of 1919 and has therefore been subjected to a considerably higher degree of inbreeding. This has apparently resulted in the isolation of a type genetically different from the wild stocks commonly collected.

Mutant Stocks.—With respect to the various mutant stocks the tests show a uniform failure to tolerate a temperature of 31°C. for more than one generation. This is more or less expected in view of the fact that most of the *Drosophila* mutants appear to be less viable than the wild stocks. This is very striking in the case of the so called II–III stock (a combination of three second chromosome

TABLE I.

Summary of Tests of Drosophila Stocks at 31° C.

Stock, source, and year collected.	No. of tests.	Result in F_1.	Result in F_2.	No. of generations carried at 31°C.
D. melanogaster				
Normal wild stocks from:				
Amherst, Mass. (H. H. Plough, 1922).	1	Normal.	Same.	8 (died).
Georgia (J. Krafka, 1922).	1	"	"	10 (").
Sweden (A.H. Sturtevant, 1922).	2	"	"	(a) 11 ("). (b) 4 (discontinued).
Florida (M. Grossman, 1922).	1	"	"	3 (").
Devil's Lake, N. D. (C. Thompson, 1920).	3	"	"	(a) 3 (died). (b) 7 ("). (c) 14 (discontinued).
Attleboro, Mass. (H. H. Plough, 1919).	3	"	(a) No offspring. (b) ± 75 " (c) ±100 "	1 (died). 2 ("). 2 (").
Mutant Stocks:				
eosin-miniature.	2	Normal.	(a) 10–15 flies. (b) No offspring.	2 (died). 1 (").
black-purple-curved.	2	(a) ±20 flies. (b) Normal.	" " " "	1 ("). 1 (").
II–III stock (black-purple-curved-hairy-scarlet-spineless).	2	(a) No offspring. (b) ±50 flies.	" "	Died F_1. 1 (died).
xple stock (scute-echinus-cut-vermilion-garnet-forked).	2	(a) ±50 flies. (b) ±20 "	" " " "	1 ("). 1 (").

vermilion-garnet-forked.	10	Normal.	No offspring	1 (died).
alternated III $\left(\frac{\text{ru D pe e}^s}{\text{h st ss}} \times \text{h st ss}\right)$.	2	"	(a) " "	1 (").
			(b) ±10 "	2 (").
III-ple (ru h D st pe ss e^s).	1	±30 flies.	No "	1 (").
D. simulans				
Woods Hole, Mass. (A. H. Sturtevant, 1922).	1	Normal.	±10 offspring.	2 (died).
Alabama (A. H. Sturtevant, 1922).	1	± 40 flies.	No "	1 (").
Scarlet eye (C. W. Metz, 1921).	2	No offspring.		Died F_1.
D. virilis			(a) Normal.	3 (discontinued).
(C. W. Metz, 1921).	2	Normal.	(b) ±15 flies.	2 (died).
D. funebris		(a) ±10 flies.	No offspring.	1 (").
Amherst, Mass. (H. H. Plough, 1921).	2	(b) No offspring.		Died F_1.
Norway (A. H. Sturtevant, 1919).	1	" "		" "
D. immigrans				
(A. H. Sturtevant, 1921).	2	" "		" "
Chymomyza procnemis			(a) ±25 flies.	2 (discontinued).
(C. W. Metz, 1921).	2	Normal.	(b) ±50 "	4 (").

mutant genes with three in the third). This stock is very weak and rather difficult to keep going at normal temperatures. It is the only mutant stock from which an F_2 generation could not be secured at 31°C. Two of the stocks gave a few offspring in the F_2 generation but these flies would not breed. In the case of the mutant types the failure to breed seems to be simply a result of the weakened condition. The physiological difference is not specific but it is nevertheless genetic in the sense that it is one of the effects of most of the mutant genes. This point is emphasized further by the results of crossing these stocks as described below.

Different Species.—The tests of wild stocks of certain other species indicate that some are able to breed continuously at 31°C., while some, like the *D. melanogaster* mutants, will not tolerate this temperature. It is interesting to compare the geographical range of these species with the results of these tests. *D. funebris* and *D. immigrans*, according to Sturtevant,[3] are northern forms and not commonly found in tropical climates. These species show the most marked inability to breed at 31°C., usually giving no offspring even in the F_1 generation. On the other hand, *D. virilis* and *Chymomyza procnemis*, which are found in the tropics, can apparently be bred at 31°C. as well as the stocks of *D. melanogaster*. It is curious that *D. simulans*, which Sturtevant has shown to be so similar to *melanogaster*, and to parallel its range, cannot be bred at 31°C., at least as far as the available stocks are concerned. These data indicate that species differ genetically in their ability to tolerate high temperature, and that this is of some importance in determining the normal range of the species.

Experiments at Higher and Lower Temperatures, Indefinitely and for Short Periods.—A number of tests indicate that 31°C. is the highest temperature at which any of the *Drosophila* cultures can be bred. Most of the stocks which will go at 31°C. were tried at 32 and 33°C., and while a few gave offspring for one generation, these could never be carried further. At these temperatures the bacterial growth in the food is very active, the agar usually becomes partly liquified, and the bottle goes bad. However, the fact that flies do, now and then,

[3] Sturtevant, A. H., North American species of *Drosophila*, *Carnegie Inst. Washington, Pub. 301*, 1921.

come through, indicates that this is not the cause of the failure of the culture.

We have confirmed Northrop's finding that there is no evidence of hereditary adaptation to high temperature. Stocks which will breed indefinitely at 31°C. cannot be bred at 33°C. even after six to eight generations of continuous exposure to the lower temperature.

At a temperature of 29.5°C. most of the cultures will breed with difficulty. In several tests the black-purple-curved stock and the eosin-miniature stock failed after the first generation. On the other hand the vermilion-garnet-forked stock was carried for four generations at this point without difficulty, before being discontinued. The wild Attleboro stock was also carried at this temperature for the same period, but the number of offspring was small and difficulty was experienced in keeping it going. The wild *D. funebris* and *D. immigrans* stocks which showed marked inability to tolerate 31°C. gave larvæ and pupæ at 29.5°C., but no imagos hatched out. This temperature therefore appears to be close to the critical temperature for stocks which will not go at the higher point.

Northrop reported that his stock of *D. melanogaster* could be carried indefinitely at 30°C. if the cultures were brought back to the optimum temperature for a period of 24 hours or more during each generation. We find that the stocks which will not go at 31°C. will recover from the effects of exposure to it for one generation, but the period at lower temperature necessary for recovery is longer. Most of the mutant stocks will breed again at 31°C. if returned to 24°C. for about 4 or 5 days. The vermilion-garnet-forked stock gave a small number of offspring in the second generation at 31°C. when the F_1 flies were returned to 24°C. for 72 hours. On the basis of such observations Northrop concluded that the eggs of the flies kept at high temperatures were injured, but that normal eggs would be produced if the parents were returned to normal temperatures and these normal eggs would develop at high temperature. No proof of this idea was given and our crosses of flies exposed to high temperatures with control flies shows it is by no means an adequate explanation.

Extraction and Crosses.—Since the inability to breed at 31°C. seems to be an inherited character, a number of crosses were made

between stocks which would go at 31°C. and those which would not, in order to determine the behavior of the gene or genes which are responsible for it. Two such cases where mutant characters are involved may be cited. The Sweden wild stock was crossed to the black-purple-curved, and the Georgia stock to the sex multiple. In both cases the normal heterozygous stock would breed indefinitely at 31°C. like the wild parent, but extracted flies, showing any of the mutant characters, failed to breed at this point although they went perfectly well at the control temperature of 24°C. Some twenty-five tests of this sort, with three pairs in each bottle were made in an attempt to discover whether the reaction to temperature was due to a separate gene which would segregate from the mutant genes. The negative result indicated that the inability to withstand high temperature is not separable from the mutant genes and is simply one of the physiological effects of the presence of the mutant genes themselves. As suggested above, these characters all produce a weakening, which, if for no other reason, would probably prevent their survival in a tropical climate.

With regard to crosses of the single wild *D. melanogaster* stock which refused to breed at 31°C., the Attleboro stock, our evidence is still inconclusive. This stock crossed to Amherst wild stock gave normal offspring at 24°C. and the hybrid stock will breed indefinitely. The same is true of crosses made to any of the mutant types at 24°C. When the Attleboro by Amherst cross is made at 31°C., normal F_1 flies hatch. Six bottles of these F_1 flies with two pairs each failed to give any offspring at 31°C. although many larvæ appeared in the bottles. The same cross at 24°C. went without difficulty. While further confirmatory tests are necessary, this result suggests that a dominant gene or genes in the Attleboro stock produce failure to tolerate a temperature of 31°C.

Evidence of the Effect of High Temperature on the Germ Cells from Crosses.—The nature of the effect of the high temperature on those flies which will not breed is an interesting question. Normal flies are to be expected for one generation since the germ cells which form the F_1 are matured at the normal temperature before the exposure. In cultures of the F_1 flies hatched at 31°C. and continued at that temperature, copulation takes place and a large number of eggs

are laid. In all cultures at high temperatures more eggs are laid on the glass above the food than in cultures at normal temperatures. In spite of their normal appearance, practically none of these eggs give rise to larvæ. The few which do give rise to larvæ and imagos, whether of the Attleboro or any mutant stock, can never be carried any further.

We have examined sections of the gonads of flies newly hatched at 31°C., and except for their small size, they are similar to the gonads of normal flies of the same age. Both mature eggs and sperm are present.

We then tried crossing males and females from susceptible stocks kept at 31°C. for one generation to females and males hatched at 24°C., placing the bottles at the former temperature. If the germ cells of either were injured then no offspring could be expected, just as in the case where the F_1 flies were mated in mass cultures. The results are shown in the following table. The mutants being sex-linked, all classes hatched out in the F_2 generation; the heterozygous flies could be bred for an F_3 generation but those having mutant characters would not go.

TABLE II.

Crosses Made at 31°C.

F_1 flies hatched at 31°C.		F_1 flies hatched at 24°C.	Total No. of bottles started.	No. from which F_2 hatched.
vermilion-garnet-forked ♀	×	Amherst wild ♂	7	7
vgf ♂	×	" " ♀	7	5
" ♀	×	Attleboro wild ♂	1	1
" ♂	×	" " ♀	1	1
" ♀	×	vgf ♂	5	3
" ♂	×	" ♀	3	0
		F_1 flies hatched at 31°C.		
		(From non-susceptible stock.)		
vgf ♀	×	Devil's Lake wild ♂	2	2
		(From susceptible stock.)		
" ♀ and ♂	×	vgf ♂ and ♀	23	0

These data show clearly that while susceptible stocks will not go for more than one generation at 31°C. when inbred, both males and females will produce normal offspring at this temperature when

mated to other flies which have hatched at a lower temperature or to flies which breed normally at 31°C. Clearly, then, neither the eggs nor the sperm are injured by the exposure to 31°C. since they perform their function normally enough in crosses. The reason for the failure to produce offspring after the first generation seems to lie either in a failure of the sperm to reach the eggs—that is unsuccessful copulation—or in the failure of the fertilization process itself. This problem is now under investigation and we cannot as yet answer the question. As noted above F_1 flies hatched from these heat-sensitive stocks copulate normally as far as outward appearances indicate at 31°C., so that it would appear that fertilization was not successful. It has not been determined whether sperm are actually present, however, in the seminal receptacles of the females.

SUMMARY.

1. Most wild stocks of *Drosophila melanogaster* can be bred indefinitely on banana agar at a temperature of 31°C. There is no relation between the geographical origin of these stocks and their ability to tolerate this temperature.

2. A single wild stock has been found which will breed for only one generation at temperatures above 29°C. The offspring hatched at 31°C. will breed normally at 24°C. This difference from other wild stocks is apparently genetic, but its genetic basis has not yet been worked out.

3. The mutant stocks of *D. melanogaster* tested by us will breed for only one generation at 31°C. and their offspring at this temperature are also fertile at 24°C. This condition is apparently a physiological effect of the presence of any of the mutant genes in a homozygous condition.

4. Similar tests indicate that wild stocks of *D. virilis* and *Chymomyza procnemis* will breed at 31°C., while *D. simulans*, *D. immigrans*, and *D. funebris* will not. The last two species are northern forms not commonly found in the tropics.

5. Both male and female flies from mutant stocks hatched at 31°C. produce offspring at this temperature if mated to flies hatched at 24°C. Their germ cells are therefore capable of development, and the cause of their failure to develop at 31°C. when inbred must lie either in the failure of the germ cells to reach each other or in the fertilization process itself.

ELECTROPHORESIS OF BACTERIA AS INFLUENCED BY HYDROGEN ION CONCENTRATION AND THE PRESENCE OF SODIUM AND CALCIUM SALTS.*

By C.-E. A. WINSLOW, I. S. FALK, and M. F. CAULFIELD.

(*From the Department of Public Health, Yale School of Medicine, New Haven.*)

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I.

INTRODUCTION.

Since the fundamental work of Hardy (1899, 1900) (18, 19) a number of investigators have reported experimental results directly or indirectly bearing upon the electrophoretic charge of the bacterial cell. These studies indicate clearly that in solutions at or near the neutral point bacteria commonly migrate to the anode in an electrical field. (2, 26, 39, 6, 7, 32, 3, 34, 1, *a*, 20, 33, 16, 35, 36, 37, 31, 29.) Only Thornton (40) and Abbott (1), report conflicting results. Yeasts also carry a negative charge (20, 37) while one author (7) describes a mold, viable in high concentrations of acid, carrying a positive charge. Results with spirochetes, trypanosomes and other protozoa are diversified (37, 31, 41, 21). Heat-killed bacterial cells exhibit in general the same electrophoretic phenomena as living cells (7, 32, 1, *a*, 33, 16, 31). An increase in hydrogen ion concentration tends to diminish the electrophoretic charge and an isoelectric point is found near pH = 3.0 (39, 32, 1, *a*, 33, 16, 31, 29). A positive charge may under certain circumstances, be developed beyond this point (39, 1, *a*, 29). The presence of other electrolytes also exerts a marked influence upon electrophoresis, depending upon concentration and upon the free valency and nature of the ions concerned (39, 32, 20, 16, 35, 36, 37, 29)—particularly in the case of ions bearing a charge opposite to that of the bacterial cells.

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II.

Technique.

The experiments here reported[1] were undertaken to obtain comparable data in regard to the influence of various hydrogen ion concentrations in water and in sodium and calcium chloride solutions (over a wider range than had hitherto been studied) upon the electrophoresis of a large bacillus (*Bacillus cereus*). The bacteria were grown in Kolle flasks at 37°C. (except when otherwise stated in the specific protocol of an experiment). Growth was removed with a small volume of water or other test fluid by a gently rotary motion of the culture flask. The suspension was then shaken vigorously in a mechanical shaker or by hand to break up clumps of bacteria and to give a uniform distribution of the organisms through the menstruum. The organisms were then washed by repeated precipitations in a centrifuge at some 3,000 revolutions per minute and taken up each time in the fluid in which the electrophoresis was to be measured.

The test fluids were prepared in carefully distilled water using pure salts and pure acid and alkali. To prepare a suspension of bacteria in water or in a salt solution at a particular hydrogen ion concentration our practice was to suspend the bacteria in the water or salt solution and then to adjust to the required pH by adding the necessary amount of acid or alkali as determined by the titration of an aliquot portion of the suspension. Such a titration was accomplished by adding to a 5 or 10 cc. fraction the appropriate indicator and adding acid or alkali to the required pH as measured colorimetrically against solution color standards or—more commonly—against standard color plates (Clark, 8). When adjusting solutions to the extremes of the pH range we obtained more satisfactory results by conducting the titrations electrometrically with the hydrogen electrode, using the titration chambers briefly described by Falk and Shaughnessy (13). Adjustments of pH which were made colorimetrically were occasionally checked electrometrically and found to be satisfactorily precise for the purposes of the experiment. No buffer substances were added to our test fluids because of the desire to avoid the single or

[1] Certain of these experiments have already been presented in a preliminary form (45).

mutual effects of salts other than those specifically studied. That the phenomena studied may be seriously modified by the presence of buffer salts is well illustrated by the experiments of Beniasch (3). He failed to obtain acid agglutination of *Bacterium coli* in the presence of buffer salts. Eggerth and Bellows (9) avoided this salt interference by conducting their experiments without the buffer salts. This procedure necessarily introduces an undesirable instability of the pH (except where the salt content of the fluid was sufficiently high to serve as an effective buffer or where the pH was so high or so low as to be unaffected by the buffering powers of the bacteria) which necessitates a check reading of the pH *after* the electrophoretic manipulation. (The tendency of bacteria to adjust the pH of their menstrua has been discussed at length by Winslow and Falk (43, 44).) Our measurements were conducted at room temperature.

The measurement of the velocity of migration of *Bacillus cereus* in any particular fluid under the influence of an impressed voltage was made by the direct microscopic method described by Northrop (27) with certain modifications courteously demonstrated to us by Dr. Jacques Loeb. These modifications consist in the addition of a pair of funnels connected through stop-cocks to the vessels containing the zinc-zinc sulfate electrodes. The design of this cell permits of the application of a direct current potential through non-polarizable electrodes and of the observation under a magnification of 525 diameters of the velocity with which any individual bacterial cell moves towards the positive or negative pole. The heating effect of the lamp illuminating the field of the microscope was controlled by the interposition of a flat walled flask containing cold water. The basic theoretical and technical aspects of such an apparatus are thoroughly treated by Ellis (10, 11), Powis (30), and Northrop (27) and need not be repeated here. Inasmuch as there is a potential difference between the surfaces of the liquid and the glass,[2] upon application of an external potential there is an electrical-endosmose movement of the liquid. The cell as a whole, however, is a closed system. Therefore, the total movement of the water must be zero. That is, the water which moves towards the cathode at the glass surfaces returns towards the anode in the midregions of the fluid in

[2] Water is electropositive to glass.

the cell. Obviously, then, the electrophoretic movement of the particles will be impeded at certain levels in the cell and accelerated at others by the independent movement of the water. In addition, of course, there is the electrical-endosmose due to the difference in potential between the water and the particles. The true mobility of the particles is the average mobility at all levels in the cell. We have followed Northrop's modification of the technique of Ellis in making measurements at the levels corresponding to the middle of each sixth of the distance between the under surface of the cover-glass and the upper surface of the base of the cell and taking the average of these values as the true mean velocity of the bacteria.[3] Calculation of the potential fall per cm. in the cell (X in the Helmholtz-Lamb equation) was made by the usual method (*vide* Northrop, 27).

The results of our experiments are expressed throughout in terms of actually observed velocity of migration (in micra per second). Observations were made by the use of an ocular micrometer and stopwatch, each figure representing the mean of at least fifteen observations, one-third at each level. Where movement in opposite directions was apparent at different levels an average velocity was obtained for each level and the three level values were then algebraically averaged. It would be eminently desirable to convert the velocity figures into terms of electrical potential difference at the interface between the bacterium and the menstruum. If we adopt the Helmholtz-Lamb equation (Burton, 4, 5) this value can be found from the following formula.

$$V \cdot \frac{l}{d} = \frac{4\pi}{K} \cdot \frac{\eta v}{X}$$

in which

V = P.D., the difference of potential between the solid particle and the liquid.
l = a linear magnitude measuring the "slip."
d = the distance between the plates of an air condenser equivalent to that virtually formed by the opposed surfaces of solid and fluid.
K = the specific inductive capacity (dielectric constant) of the liquid.
η = the coefficient of viscosity of the liquid.
v = the mobility (velocity of migration) of the particle.
X = the strength of the electrical field,
all measurements being made in electrostatic units.

[3] Inasmuch as the upper and lower halves of the cell are symmetrical we dispensed with three readings at the mid-levels of each third of either the upper or lower half of the cell.

Although the form of the Helmholtz-Lamb equation is simple, its use in precise experimental work involves a number of difficulties because of the uncertainties attached to some of the terms involved. Thus, finite values for d and l are not at present known. But because there is some justification for considering that—under certain conditions, at least—the ratio $d:l$ approximates unity, it is sometimes the practice to omit these terms in calculating the P.D. (potential difference) between particles and a liquid. Another procedure is to refer to the calculated value of $(V \frac{l}{d})$ in volts as the potential difference. We are then dealing, however, with the Helmholtz and not the Helmholtz-Lamb equation. If one accepts as valid the theoretical considerations upon which Lamb's calculations were based and yet disregards $l:d$, obviously the P.D. calculated from experimental values is —in a *quantitative* sense—open to considerable question.

A more serious difficulty which is often overlooked, is the uncertainty which attaches to K, the dielectric constant for the solution. This constant should, theoretically, apply to the specific inductive capacity of the medium which separates the positive and negative layers of electricity on the particulate and liquid phase surfaces. When dealing with a pure menstruum free from solute and in which the substance of the suspended particles is imperceptibly soluble we can conceive that the dielectric constant of the liquid, as ordinarily determined, may be applied in the equation although the accuracy of this procedure is not clearly known. When dealing with a liquid in which the particles themselves are appreciably soluble or in which other substances have been dissolved, the case may be significantly different, depending upon whether the dissolved substances do or do not appreciably affect the dielectric constant of the liquid between the Helmholtz double layer. Even when the effect of the solute upon the inductive capacity of the bulk of the liquid is known, it must still remain an open question what the effect of the solute is upon the thin film of liquid which is conceived as lying between the electrical layers. Unfortunately the influences of electrolytes upon the dielectric constant of water are far from being clearly established. The subject was reviewed recently by Lattey (23). He points out that certain earlier investigators came to diametrically opposite conclusions on this

problem. Thus, Drude, Coolidge, and others were unable to distinguish between the dielectric constants of pure water and of solutions of copper sulfate. Smale, on the other hand, asserted that an 0.05 N solution of this salt showed a dielectric constant 15.5 per cent greater than that of water. Smale's view is supported (qualitatively, at any rate) by the data of Walden (42). For aqueous KCl solutions (1,000, 200, and 100 liters per mol concentration) the dielectric constants *increased* in his experiments from 81.7 for pure water to 82.8, 84.5, and 90.9, respectively. Also, Walden calculated the dielectric constants for the dissolved salts and obtained values far higher than those usually obtained for the solid, undissolved salt. Walden's conclusion was that with dissolution of electrolytes in solvents in which ionization occurs, the dielectric constant of the solvent is appreciably increased. Other investigators are similarly divided in their conclusions. Cohn, Yule, and others are in accord with Walden in having found *increases* in the dielectric constant of water with solution of electrolytes; Nernst, Palmer, and others, like Drude, found no significant changes with small concentrations of salts; and others have obtained results similar to those of Lattey, the most recent observer, who reports *decreases* in the dielectric constants of salt solutions. Lattey (23) himself presents a review of the methodology of earlier workers and finds ground for questioning the validity of many of their findings. For pure water he takes as a mean value for the dielectric constant the value 81.05. His data for aqueous solutions of potassium chloride (from 0.000755 to 0.00755 N) clearly show *depressions* of the dielectric constant. The depression is greater the higher the salt concentration and reduces the constant to 66.25 (81.5 per cent of the constant for water) in the most concentrated of these dilute KCl solutions. The data for more concentrated KCl solutions were not published because of uncertainty which attached to their accuracy. Similarly for aqueous copper sulfate solutions of 0.00114 to 0.00456 normality the dielectric constants were lower than for pure water. In the most concentrated solution the depression amounted to 10 per cent of the absolute value of the constant for pure water. In concluding, Lattey remarked that: "The electrolytes investigated appear to lower the dielectric constant of water, and are in this respect analogous to the majority of non-

electrolytes." All in all it appears that no unambiguous conclusion concerning the value of K in the Helmholtz-Lamb equation when studying electrophoresis in electrolyte solutions is possible. The changes in K produced by electrolytes which have been reported do not, in general, exceed $\pm$ 10 per cent of the K for water. They must, however, be taken with the precaution that only comparatively dilute solutions have thus far been carefully studied. The experimental difficulties inherent in studies upon concentrated solutions are not easily avoided. Until the values of K for various solutions are made known with some precision, the usefulness of the Helmholtz-Lamb equation in biological work will be markedly limited.

Besides K, η, the coefficient of viscosity of the fluid, is a variable in the Helmholtz-Lamb equation and its value demands some consideration here. In the papers reported by Northrop and his colleagues, K was taken as 80, η as 0.009 and because of the uncertainties attached to their evaluation, neither was treated as a variable although the solutions studied were of various hydrogen ion concentrations, and contained buffering salts and various concentrations of certain salts whose influences on P.D. were being investigated. (Northrop and Cullen, 28.)

According to the data cited in Tables annuelles de constantes (38), η increases markedly with salt concentration. Thus, for aqueous solutions of sodium chloride the coefficient of relative viscosity (η_{25} (rel))[4] increased from 1.118 to 1.862 with increasing concentrations of the salt from 1.25 to 5.0 mols per liter and for calcium chloride the absolute coefficient of viscosity at 15°C. (η_{15}) increased from 0.01528 to 0.11709 with increasing concentrations of the salt from 123.5 to 580 gm. per 1,000 cc. of solution. According to the data cited by Kaye and Laby (22) normal solutions of certain electrolytes show markedly increased viscosity as compared with pure water, others show no appreciable change, and others (*i.e.* NH_4Cl, KI) show diminished viscosity. In our studies we have given especial attention to the chlorides of sodium and calcium and it is therefore significant to note that the extent of the changes which these electrolytes cause is appreciable and varies with the concentration of salt. Obviously

[4] The coefficient of viscosity of the solution at 25°C. relative to the coefficient for water at the same temperature.

these effects of the electrolyte cannot be overlooked in calculating P.D. from v in the Helmholtz-Lamb equation. Burton (4, p. 145) has clearly shown the significance of variations in the viscosity of water in relation to the mobility of colloidal silver particles. The product ηV, he found, is sensibly constant. In conclusion from these considerations it seems to us that it is a highly questionable procedure to convert measured mobilities of particles (v) into P.D. $\left(V\frac{l}{d}\right)$ unless the variables involved are carefully controlled or measured.

On account of these uncertainties we have preferred to express our results in terms of observed velocity. If, however, we write $V\frac{l}{d}$ = P.D. and if we assume that the dielectric constant, K, is invariably 81 and that for pure water the coefficient of viscosity at 20° is equal to 0.010 our formula becomes

$$\text{P. D.} = \frac{4\pi}{81} \cdot \frac{0.010\, v}{X} \text{ (in C. G. S. units).}$$

The dimensions of our apparatus were such that the potential gradient per cm. (X) in the electrophoresis chamber was 0.106 times the impressed voltage (11.7 volts for the voltage of 110 generally used). To convert this value to C.G.S. units it must be divided by 300 and to express the final result in practical units we must multiply the whole fraction by 300.

Thus

$$\text{P. D.} = \frac{4\pi}{81} \cdot \frac{0.010\, v}{\frac{11.7}{300}} \cdot 300 \text{ (in volts).}$$

Whence

$$\text{P. D.} = 12\, v \text{ (in volts).}$$

Here v is in cm. per second. If v is expressed in micra per second,

$$\text{P. D.} = 1.2\, v \text{ (in millivolts).}$$

Our results in micra per second may be multiplied by this factor to give what may be considered to be a probable figure for absolute potential difference; and it may be noted that the figures thus derived are in reasonable accord with those given by Girard and Audubert

(16), Shearer (35, 36), and Northrop and De Kruif (29). They are somewhat lower, however, than the potential differences obtained by Northrop and De Kruif who report that a P.D. of 15 millivolts represents the critical point below which various bacteria agglutinate while it will be noted that our results indicate a charge in distilled water of neutral reaction equal to about 11 millivolts (averaging values in Table I for pH 6.0 to 7.9 and multiplying by 1.2). Whether this means that *Bacillus cereus* has a lower charge than the organisms studied by Northrop and his colleagues, or whether the difference is due to the complex reactions which occur in the zone surrounding a bacterial cell in an unbuffered solution, it is impossible to say.[5]

It should be noted that the P.D. value computed as above is the *electrokinetic* P.D. whose existence is evidenced in electrophoresis, or electro-osmose, or Poiseuille streaming experiments and which is most readily understood in terms of the Helmholtz electrical double layer concept. This must not be confused with the Nernst electromotive or so called *thermodynamic* P.D. between the inside of one phase and another in contact with it (*i.e.* water and glass) (17, 14, 15).

III.

Electrophoresis of Living Vegetative Cells at Various Hydrogen Ion Concentrations.

The general type of data obtained in the present study may first be indicated by citing a few typical experiments. Experiment 12, for example, gave the figures indicated below for a hydrogen ion range pH 1.0 to 10.0, inclusive.

Experiment 12.

pH.....	1.0	1.5	2.0	2.6	3.3	3.7	5.5	6.8	7.2	7.3	7.7	9.6	10.0
Velocities in micra per sec.....	0	+2.8	+2.5	+1.9	−1.8	−8.9	−9.7	−7.2	−10.6	−10.3	−10.3	−8.7	−10.0

+ signifies rate of movement toward cathode, − toward anode.

[5] The paper by Eggerth (Eggerth, A. H., *J. Gen. Physiol.*, 1923, vi, 63) published since this manuscript was prepared, suggests another possible explanation for this difference.

The suspension was at a pH of 6.8 when first tested. With decreasing hydrogen ion concentration (up to pH 10) no marked change in velocity of migration was apparent, the bacteria in each case moving toward the anode at a rate between 7 and 11 micra per second. An increase in acidity below pH 3.7 was on the other hand attended by a marked decrease in electrophoresis, with a reversal of movement (toward the cathode) at pH values between 1.5 and 2.6. An isoelectric point at about pH 3.0 is indicated and below pH 1.0 the velocity again returns to approximately zero.

Experiment 19 illustrates the phenomena observed within the alkaline range.

Experiment 19.

pH	8.8	9.8	10.4	10.9	11.5	11.6	12.2
Velocity in micra per sec.	−12.2	−20.0	−19.2	−23.8	−13.5	−6.3	−17.2

Here we see that at alkaline ranges between pH 9.8 and 10.9 the velocity of migration is markedly increased. Above pH 11 it falls again (in some experiments approximating zero) while in even more highly alkaline solutions it may perhaps show a second rise, although our data here are too few to warrant definite conclusions.

One more experiment may be cited in detail, Experiment 10, in which we modified our usual technique by washing off our original suspension in water at pH 2.9 and rewashing three times in water of the same pH in order to begin with a suspension of bacteria approximately isoelectric with their menstruum.

Experiment 10.

pH	1.0	1.5	2.2	2.9	3.4	3.7	4.6	5.1	6.2	8.1	10.0
Velocity in micra per sec.	+1.2	+2.7	+3.8	+2.7	+3.1	−0.7	−13.1	−9.4	−11.0	−13.6	−13.1

The original suspension (pH 2.9) was evidently not exactly isoelectric since the bacteria moved with a considerable velocity toward the cathode. At more acid reactions this velocity first increases and then diminishes. At less acid reactions the migration toward the

TABLE I.

Observed Velocities (in Micra per Second) of Living Vegetative Cells at Different Hydrogen Ion Concentrations (All Experiments).

pH	1.0 or less	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9	11.0–11.9	12 and over
No. of observations	4	4	8	8	3	7	13	5	8	5	11	8	5
Minimum velocity*	+1.2	+2.8	+3.8	+3.1	−6.6	−7.2	−3.6	−7.8	−7.7	−8.7	−7.0	0.0	0.0
Maximum velocity	−4.5	+0.1	−10.6	−8.9	−13.1	−15.4	−13.1	−10.6	−17.2	−20.0	−23.8	−13.5	−18.2
Average velocity	−0.8	+1.7	+0.3	−2.8	−8.8	−10.8	−8.7	−9.7	−12.0	−13.8	−15.6	−6.3	−10.2

* For convenience all velocities are here expressed with the anode as a point of reference. Therefore, at pH values below 4.0 the "minimum velocity" included in the table is the *maximum* velocity toward the cathode.

cathode is reversed and above pH 4.5 we observe a normal movement toward the anode.

The general relations observed may best be indicated by the averages in Table I.

As in the study of most biological phenomena differences were manifest in the results of different experiments, due to unavoidable pH variations in unbuffered solution, to slight differences in room temperature and in the biological characteristics of the oganisms, or to other unknown factors. On the whole, however, the lack of extreme variability was rather encouraging except in the case of the observations at the extreme alkaline end of the range. It seems on the whole clear that the bacteria studied moved toward the anode with a high and fairly uniform velocity at hydrogen ion concentrations on the alkaline side of pH 4.0. As we pass into the alkaline zone between pH 8.0 and 11.0 there is a definite increase in velocity and beyond pH 11.0 there is a second fall, reaching an isopotential point in individual instances. At the extreme alkaline end of the range (beyond pH 12.0) results are highly variable but we have not yet sufficient data to draw definite conclusions as to the course of phenomena in this zone.

At pH values below 4.0 there is a very sharp drop in velocity with an isoelectric point at about pH 3.0. At more acid reactions the direction of migration is reversed, being definitely toward the cathode between pH 1.0 and 2.0, with another trend toward diminished and variable migration at pH values below 1.0, tending again to approximate a condition in which the bacteria are isopotential[6] with their menstruum.

IV.

Electrophoresis of Killed Vegetative Cells at Various Hydrogen Ion Concentrations.

Two sets of experiments were made with suspensions prepared in the same manner described above but heated in an autoclave for 20 minutes at 17 pounds pressure to kill the bacteria. The results are indicated in Table II.

[6] The "isopotential" zones at the extreme acid and alkaline ranges probably are not true isoelectric points in the same sense as the pH 3.0 zero potential point of inflection in the electrophoresis curve. We therefore prefer to use the word "isopotential," suggested by Dr. Stuart Mudd.

With only two experiments random errors are likely to be considerable; but it is evident that the heat treatment has not materially affected the electrophoretic charge of the bacterial cells. The values are essentially the same as those recorded in Table I. The normal rate of migration toward the anode manifest near the neutral point rises as the solution is made more alkaline (pH 9.0 to 9.9) and falls as it is made more acid, almost disappearing at pH values near 3.0.

It is of considerable interest to note that the killing of bacterial cells by heat does not substantially alter their electrical charge since it suggests (if the phenomena of potential difference are to be explained on the theory of the Donnan equilibrium, to be discussed later on) that the permeability of the cell surface to those ions concerned in potential difference has not been materially affected by this treatment. The observations are in accord with the results of earlier workers

TABLE II.

Observed Velocities (in Micra per Second) of Killed Vegetative Cells at Different Hydrogen Ion Concentrations.

pH	1.0 or less	2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	7.0-7.9	8.0-8.9	9.0-9.9
Velocity, Exp. 7*a*		−0.2		−4.0		−4.4		−6.0
Velocity, Exp. 9	−2.8		−1.7		−8.3	−14.2	−9.4	−17.0

(7, 32, 1, *a*, 33, 16, 31, 29) and with the familiar fact that the killing of bacterial cells by heat (as in the pasteurization of milk) does not alter their staining properties.

It should be noted that in our experiments on so called living vegetative cells described above many of the bacteria must actually have been killed by the extreme acidities and alkalinities used. It is probably of real significance that the hydrogen ion zone of migration toward the anode is the zone within which bacterial life is ordinarily possible and that the zones of isopotential and migration toward the cathode (whether conditioned by acids, alkalies, or salts) are beyond the limits of normal viability. In other words marked deviation from ordinary potential differences is very probably incompatible with viability. On the other hand the death of the cells need not alter their electrophoretic reaction as evidenced not only by our

studies of heat-killed cells but also by the fact that bacterial cells whose migration has been reversed in direction by exposure to pH 1.0 exhibit a quite normal migration toward the anode when brought back to the neutral zone.

V.

Electrophoresis of Bacterial Spores at Various Hydrogen Ion Concentrations.

In studying the electrophoresis of the spores of *Bacillus cereus* two different methods were used. In our first experiments old agar slants, already rich in spores, were washed off and tested directly. In later work 18 hour agar cultures were washed off and held in water suspension, until sporulation had occurred. Both methods proved satisfactory, vegetative cells being rarely observed in either case.

The general results of these experiments are indicated in Table III.

TABLE III.

Observed Velocities (in Micra per Second) of Spores at Different Hydrogen Ion Concentrations.

pH.....	1.0–1.9	3.0–3.9	5.0–5.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9	11.0–11.9	12.0 and over
Velocity, Exp. 20.....				−5.0	−10.9	−11.8	−16.0	−13.5	+10.9
Velocity, Exp. 38.....	+2.9	−0.7	−3.4	−5.9	−6.8	−8.4	−9.4		
Velocity, Exp. 39.....	+1.7	0.0	−2.8	−4.7	−6.6	−5.8	−9.8		
Average velocity.....	+2.3	−0.3	−3.1	−5.2	−8.1	−8.7	−11.7	−13.5	+10.9

These results are even more strikingly concordant than those obtained with the vegetative cells and the spores behave on the whole exactly as the vegetative cells did, although the absolute velocity appears to be slightly less at all pH values. That the velocity of spores and vegetative cells is so nearly identical and if anything slower, in the case of spores, is in accord with the theoretical physical considerations of the Quincke-Helmholtz-Lamb analysis which postulates that electrophoretic velocity is independent of the mass of the moving particle.

Again we note an isoelectric point in the neighborhood of pH 3.0, with migration toward the cathode at more acid reactions and with migration toward the anode at less acid reactions, increasing in

velocity up to pH 11.0. The positive charge manifest in a single experiment at pH 12.0 may or may not be significant. This is a point to which we are now giving further study.

VI.

Influence of NaCl upon Electrophoresis of Bacterial Cells.

Our next experiments were designed to test the influence of sodium chloride upon the electrophoretic charge. The material was prepared as described above except that the bacteria after suspension in water were centrifuged and resuspended two or three times in the salt solution in which their migration was to be observed. When the reaction of the salt solution diverged appreciably from neutrality

TABLE IV.

Observed Velocities (in Micra per Second) of Living Vegetative Cells at Different Hydrogen Ion Concentrations and in the Presence of Different Concentrations of NaCl.

pH	1.0–2.0	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9
0.145 M NaCl	+0.9	+0.8		−3.4		−4.9		−4.0	0.0	0.0
0.363 M NaCl	0.0		−2.8		−6.5		−4.9	−1.8		0.0
0.725 M NaCl	−0.5	−2.7	−2.6	−3.8	−3.9	−5.1	−4.9	−2.4	0.0	0.0
1.450 M NaCl	+1.0		+0.4	−3.2		−5.6	−4.9		0.0	0.0

it was brought back to pH 7.0 before use. The results presented in Table IV are based on a single experiment at the 0.145 M, 0.363 M, and 1.450 M concentrations. For the 0.725 M concentration four separate experiments have been averaged. The correspondence between these four experiments was remarkably close.

It is evident, as pointed out by earlier workers (39, 32, 20, 16, 35, 36, 31, 29) that the presence of salts has a marked depressing effect upon electrophoretic velocity. Within the limits here studied (0.145 M to 1.450 M) this effect in the case of NaCl appears to be independent of concentration. At reactions near neutrality the velocity of migration is but little more than half as great as that observed in the absence of the salt (*cf.* Table I). The isoelectric point at about pH 3.0 is again manifest. On the alkaline side of the neutral point the most marked

effect is apparent. The rise to a maximum velocity near pH 10.0 observed in aqueous solution is here replaced by a complete absence of migration. This observation in regard to the narrowing by salts of the pH zone within which electrophoresis occurs is, so far as we are aware, a new one. Decreased rate of movement in the alkaline range may be in part explained by increase in viscosity and decrease in inductive capacity but it seems improbable that complete loss of movement can be wholly attributed to these effects of NaCl upon the menstruum without assuming a real decrease in electrokinetic potential difference.

VII.

Influence of $CaCl_2$ upon the Electrophoresis of Bacterial Cells.

We have conducted precisely similar experiments using $CaCl_2$ solutions of varying concentrations for our menstrua, the results

TABLE V.

Observed Velocities (in Micra per Second) of Living Vegetative Cells at Different Hydrogen Ion Concentrations and in the Presence of Different Concentrations of $CaCl_2$.

pH	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9
0.014 M $CaCl_2$	+6.8		−3.0		−5.5	−6.8		−3.7	0.0	0.0
0.072 M $CaCl_2$	+1.6		−3.6		−5.3		−5.6	−5.0	0.0	0.0
0.145 M $CaCl_2$	+0.8	+0.3	−1.4	−1.8	−2.3	−3.8	−3.8	−1.8	−0.4	0.0
0.725 M $CaCl_2$		0.0		0.0		0.0		0.0		

being summarized in Table V. A single experiment was made at each concentration except 0.145 M where the figures in the table represent the results of four experiments.

It appears from Table V that the effect of $CaCl_2$ in concentrations of 0.014 M and 0.072 M is almost precisely the same as that of NaCl in concentrations up to 1.450 M. An increase of $CaCl_2$ to 0.145 M however, cuts down the migration velocity very materially and a concentration of 0.725 M completely abolishes movement at all pH values. These findings are in accord with the general fact that calcium salts are more potent physiologically than sodium salts (12).

VIII.

Influence of NaCl and $CaCl_2$ When Present Together upon Electrophoresis of Bacterial Cells.

Finally, we have observed the effect of NaCl and $CaCl_2$ together when present in a ratio of four parts of NaCl to one part of $CaCl_2$, the ratio which has generally been found most favorable to bacterial viability (43, 44, 12). The actual concentrations used were 0.580 M NaCl and 0.145 M $CaCl_2$ and the average results of four experiments are presented in Table VI in comparison with pure NaCl (0.725 M) and $CaCl_2$ (0.145 M) solutions.

TABLE VI.

Observed Velocities (in Micra per Second) of Living Vegetative Cells at Different Hydrogen Ion Concentrations and in the Presence of NaCl Alone, $CaCl_2$ Alone, and Both Salts Together.

pH	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	6.0–6.9	7.0–7.9	8.0–8.9	9.0–9.9	10.0–10.9
0.725 M NaCl	−0.5	−2.7	−2.6	−3.8	−3.9	−5.1	−4.9	−2.4	0.0	0.0
0.145 M $CaCl_2$	+0.8	+0.3	−1.4	−1.8	−2.3	−3.8	−3.8	−1.8	−0.4	0.0
0.580 M NaCl + 0.145 M $CaCl_2$	−0.5	−1.2	−2.5	−3.3	−3.8	−4.4	−2.8	−1.6	0.0	0.0

The differences are very slight but there does appear to be a certain antagonistic action manifest at pH values between pH 3.0 and 6.9. Within this range a mixture of 0.580 M NaCl + 0.145 M $CaCl_2$ appears to exert a distinctly less marked depression of migration velocity than does a 0.145 M $CaCl_2$ solution alone. This finding suggests a plausible mechanism for the efficacy of antagonizing ions in stabilizing spontaneously agglutinable suspensions of bacteria in salt solution as reported by Mellon (25). It is conceivable that the antagonizing ion reduces the effectiveness of the Na^+ ion in depressing the potential difference below the critical point for agglutination of the bacteria. The theoretical possibilities involved are too complex to be discussed at the present time.

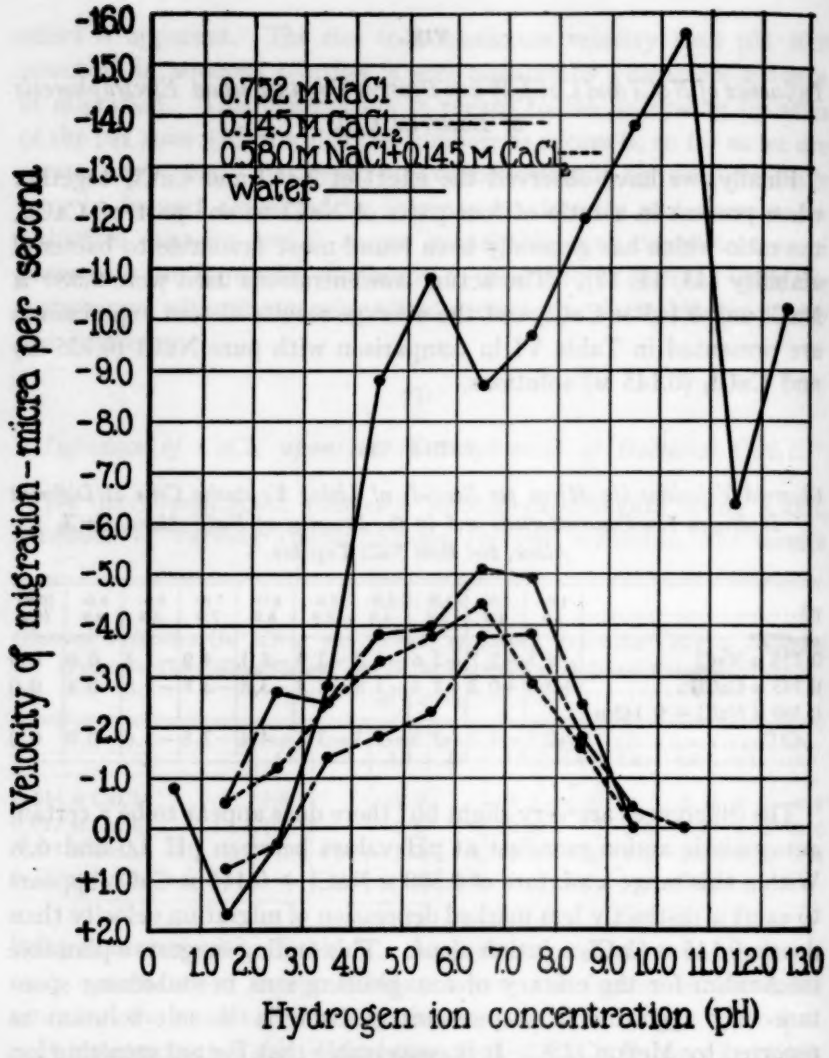

FIG. 1. The relation of the hydrogen ion concentration to the velocity of migration of living cells in water and salt solutions.

The migration velocity of −6.3 recorded at pH 11 to 12 is the average of widely varying individual observations ranging from 0 to −13.5. In this zone of hydrogen ion concentration there appears to be a sharp change from high to low velocity and probably to high velocity again, and slight variations in hydrogen ion concentration will show marked differences in result. It seems probable that, if observations were made at sufficiently close pH intervals, a point of approximate isopotential would be found in all experiments, as it was in approximately half of them.

IX.

General Theoretical Considerations.

The most plausible explanation of the differences in potential between a living cell and its surrounding menstruum is that which has been developed by Loeb (24) on the basis of the Donnan equilibrium and its application to protein systems by Proctor and Wilson.

Donnan's theory demands that when a membrane separates two solutions, the membrane being permeable to all the ionic constituents of one solution and to a part of the ionic and molecular constituents of the other solution, a definite potential difference should appear between the solutions when at equilibrium, depending on the concentrations and the charge of the various constituents. Using gelatin and other proteins as the non-diffusible constituent and various electrolytes as the diffusible constituents, Loeb shows that the quantitative results obtained in terms of potential difference are exactly what would be predicted on the basis of the Donnan equilibrium. If for example the influence of H ions on potential differences be computed, the probable variations in the dissociation constants of the substances involved taken in connection with the Donnan formula should give us a curve whose characteristics are defined by: (*a*) an isoelectric point which in the case of gelatin is near pH 4.7; (*b*) as we pass outward on the acid side of this point an area of increasing positive charge, later decreasing (with still greater acidity) toward electrical neutrality; (*c*) on the alkaline side of the isoelectric point, as we pass to less and less acid solutions the potential difference becomes increasingly negative to a maximum point and then falls toward electrical neutrality in the extreme alkaline range.

Furthermore according to the Donnan principle the addition of neutral salts should be expected, like marked excess of acid or alkali, to depress potential difference and this conclusion, too, Loeb has confirmed for compounds of gelatin and of other proteins.

As pointed out in an earlier section of this report we cannot transform our data in regard to velocity of migration into terms of potential difference between the bacterial cells and their suspending menstruum without making the assumption that viscosity and dielectric constants remain substantially constant within the range of our experimental

conditions. From computations of the known changes in viscosity in salt solutions of the strengths studied we are convinced that viscosity effects can be disregarded. That the dielectric constant also remains reasonably constant we, like other workers in this field, may for the present assume. That the phenomena involved when bacterial cells are suspended in water and salt solutions of the types studied are essentially similar to those observed by Loeb for gelatin and other types of particles and are in general accord with the postulates of the Donnan equilibrium would seem to be indicated by the general parallelism between our results and those obtained by Loeb. This parallelism has already been shown for bacterial cells by Northrop and De Kruif (29) for solutions between pH 1.0 and 9.0 but we find that in the extreme alkaline range the same parallelism is clearly manifest.

We have also confirmed the observations of Loeb and of Northrop and De Kruif that the presence of salts depresses velocity of migration, just as it should depress electrokinetic potential according to the Donnan theory and we have shown that this depression is particularly effective on the alkaline side of the pH range. We have also confirmed the findings of Northrop and De Kruif in regard to the relatively higher depressing effect of $CaCl_2$ as compared with NaCl which is another corollary of the Donnan equation.

X.

SUMMARY OF CONCLUSIONS.

1. We have confirmed the results of earlier workers particularly of Northrop and De Kruif in regard to the following points:

(*a*) the general tendency of the bacterial cell when suspended in distilled water near the zone of neutrality to move toward the anode of an electrical field;

(*b*) the fact that the migration of bacterial cells in the electrical field is a function of the reaction of the menstruum. The curve obtained by plotting velocity of migration against pH passes through an isoelectric point at about pH 3.0, at greater acidity the direction of migration becomes reversed (toward the cathode) and in still more acid solution (pH = 1.0) again disappears; while at reactions less acid than pH 3.0 the velocity is toward the anode and increases with increasing alkalinity;

(*c*) the fact that neutral salts depress the velocity of migration, calcium salts being much more effective than sodium salts of the same concentration.

2. We further find:

(*a*) that on the extreme alkaline side of the curve of velocity of migration plotted against pH a maximum value is reached at about pH 10 with a fall at about pH 12.0 which in many experiments reaches an isopotential point;

(*b*) that the depressing effect of salts is accompanied by a general shifting of the curve of migration velocity so that a maximum velocity (of course absolutely less than that manifest in the absence of salts) appears at about pH 7.0 and an abolition of velocity at pH 9.0 to 10.0;

(*c*) that an apparent "antagonistic" effect is indicated between $CaCl_2$ and NaCl, the presence of a certain concentration of the latter salt diminishing to a slight but definite degree the depressing effect produced by the former;

(*d*) that heat-killed bacterial cells exhibit essentially the same curve of migration velocity as that of the living cells;

(*e*) that bacterial spores exhibit the same general curve of migration velocity as vegetative cells, although the actual velocity is apparently slightly less.

3. All of the observed phenomena appear to be in accord with the assumption that marked differences in dielectric constants did not appear under the conditions studied and if this assumption be granted the results are in accord with the fundamental postulates of the Donnan equilibrium as applied to the explanation of the origin of potential difference between a bacterial cell and its enveloping menstruum. It is possible but not at all certain that the phenomenon of antagonism may require the introduction of additional assumptions for its explanation.

Professor Donnan and other investigators have clearly understood the importance of applying the concept of membrane equilibria in the elucidation of physiological phenomena. Our findings add to the numerous vindications favoring this view and emphasize the importance of further study of membrane equilibria in bacterial suspensions. We have pointed out that certain potential differences between

bacteria and their menstrua are apparently associated with some of the phenomena of viability. Viability and potential differences may, however, under certain conditions vary quite independently as evidenced by the fact that normal rates of migration are demonstrable after the cells have been killed by heat. Thus, considerable caution must be exercised in relating the existence of these charges to the metabolism of the cell.

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CONDUCTIVITY TITRATION OF GELATIN SOLUTIONS WITH ACIDS.

By DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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The combination of proteins with acids has been studied by Loeb[1] and others largely by means of hydrogen electrode measurements. The present work constitutes an attempt to apply the method of conductivity titration to the determination of the combining capacity of a protein for acids.

The first work which indicated that the measurement of electrical conductivity could be used in volumetric analysis seems to have been done by Kohlrausch[2] in 1885, and since then the method has been given in standard text-books.[3] Recently the method has been extensively used by Kolthoff,[4] who has outlined the theory on which it depends. In the neutralization of a weak base by a strong acid, the conductivity at first increases because of the formation of a highly ionized salt, and since at first practically all of the added acid is used up to form the salt, the conductivity increases as a linear function of the amount of acid. As more acid is added, the formation of the salt is no longer complete on account of hydrolysis, and the increase in conductivity becomes faster, due to the presence of the highly mobile hydrogen ion. Finally, after enough acid has been added to combine with all the base and to repress the hydrolysis, the conductivity increases much more rapidly on the addition of further acid, since

[1] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, Chapter IV.

[2] Kohlrausch, F., *Ann. Physik u. Chem.*, 1885, xxvi, 225.

[3] Kohlrausch, F., and Holborn, L., Das Leitvermögen der Elektrolyte, Leipsic and Berlin, 2nd edition, 1916, 136. Findlay, A., Practical physical chemistry, London and New York, 3rd edition, 1914, 199.

[4] Kolthoff, I. M., *Z. anorg. Chem.*, 1920, cxi, 1, 28, 97; cxii, 155, 165, 172, 187, 196.

practically all the added hydrogen ion remains free; and here again the conductivity increases as a linear function of the added acid. The point of neutralization is obtained by plotting the conductivity as a function of the quantity of acid added and extrapolating the two linear portions of the curve until they intersect. The shape of all the curves obtained with gelatin is similar to that of Fig. 1. Kolthoff[4] obtained curves of like shape by neutralizing phenol or boric acid with sodium hydroxide, and by neutralizing urotropin (hexamethylenetetramine) with hydrochloric acid. He thus showed that the method was applicable to the neutralization of a weak acid or base with a strong base or acid.

In applying the method to the neutralization of a protein it is necessary to consider the fact that proteins are amphoteric electrolytes. In some preliminary experiments in which gelatin solutions were first treated with sodium hydroxide and the resulting solutions titrated with hydrochloric acid, it was found that the slope of the conductivity curve exhibited practically no change at the isoelectric point. Therefore if the original gelatin solution is not at the isoelectric point it is necessary to make a correction for this in calculating the combining capacity of gelatin for acid.

The following experimental procedure was adopted. A measured volume of a solution containing a known weight of gelatin was placed in the conductivity cell, in a water bath at 33°C. ± 0.02°. The cell used was of the Freas type, holding about 130 cc. Its cell constant was not determined, since only relative conductivities were needed for this work. The conductivity was measured in the usual way with a Kohlrausch bridge and resistance box, using a small induction coil and a telephone. Standard acid was added in small amounts from a calibrated 10 cc. or 2 cc. burette, and after each addition the conductivity was measured. The results were plotted as in Fig. 1, the abscissæ being cc. of acid and the ordinates being proportional to the specific conductivities. From the intersection of the two straight lines the end-point was obtained. The pH was measured of a separate sample of each original gelatin solution, using hydrogen electrodes at 33° with a saturated KCl junction, taking as a standard the value 1.037 for the pH of M/10 HCl. The values for the end-point were corrected to correspond with an initial pH of 4.70 by adding or sub-

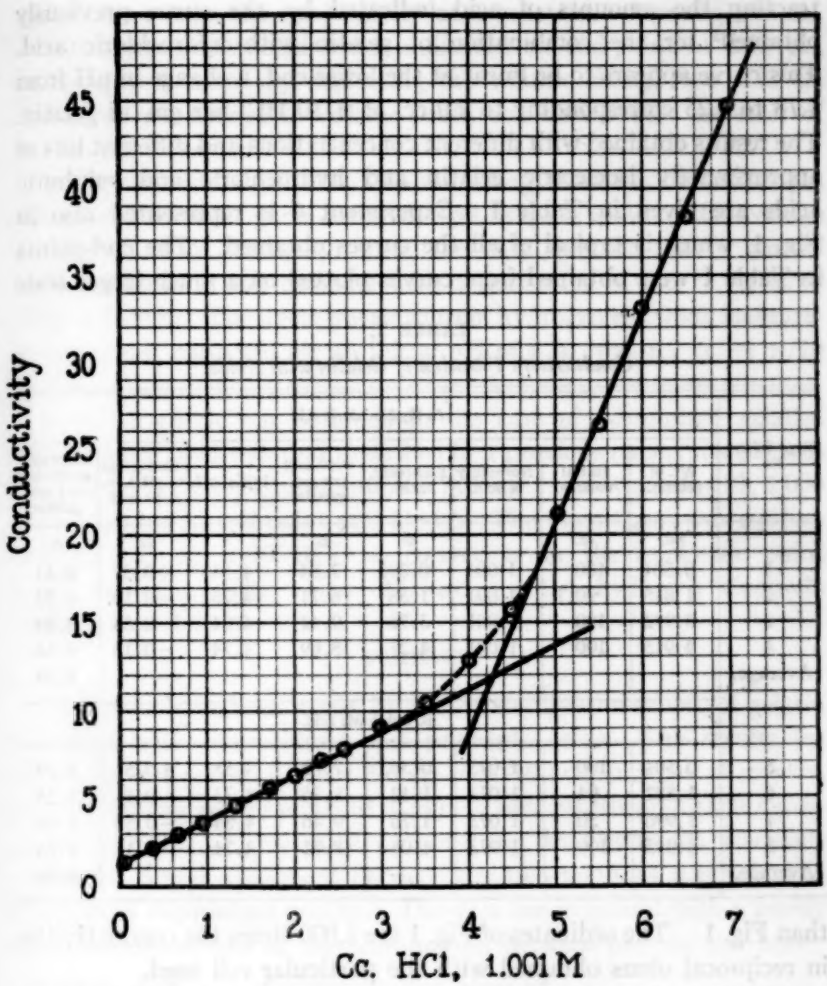

FIG. 1. Conductivity titration of gelatin with hydrochloric acid. The lower straight line represents practically complete formation of gelatin chloride from the HCl added. The curved dotted line represents the increase in conductivity due to hydrolysis of gelatin chloride. The upper straight line represents the increase in conductivity due to excess HCl, after the hydrolysis has been repressed. The intersection of the two straight lines is the apparent point of equivalence, which must be corrected if the initial pH is not that of the isoelectric point.

tracting the amounts of acid indicated by the curve previously obtained[5] for the combination of gelatin with hydrochloric acid. This curve appears to be linear at the lower end, a change in pH from 4.78 to 4.25 corresponding to 2.0 cc. of N/10 HCl per gm. of gelatin. The results obtained with different concentrations and different lots of approximately isoelectric gelatin and hydrochloric and sulphuric acids are given in Table I. Experiment 4 is represented also in Fig. 1, which is typical of all the curves obtained. The end-points in Table I were obtained from curves plotted on a much larger scale

TABLE I.

Conductivity Titration of Gelatin with Acids.

Experiment No.	Wt. of gelatin.	Initial volume.	Normality of acid.	End-point, acid.	N/10 acid per gm. gelatin.	Initial pH.	Correction to pH 4.70, N/10 acid.	N/10 acid equivalent to 1 gm. gelatin.
	A. Hydrochloric acid.							
	gm.	*cc.*		*cc.*	*cc.*		*cc.*	*cc.*
1	0.504	100	1.001	0.395	7.84	4.55	+0.57	8.41
2	1.815	90.7	1.001	1.58	8.71	4.75	−0.19	8.52
3	3.992	100	1.001	3.76	9.42	4.84	−0.53	8.89
4	4.915	100	1.001	4.27	8.69	4.74	−0.15	8.54
Average.								8.59
	B. Sulphuric acid.							
5	0.504	100	1.073	0.363	7.72	4.55	+0.57	8.29
6	1.822	94	1.073	1.42	8.36	4.73	−0.11	8.25
7	1.996	50	1.073	1.76	9.46	4.84	−0.53	8.93
8	4.915	100	1.073	4.08	8.91	4.74	−0.15	8.76
Average.								8.56

than Fig. 1. The ordinates of Fig. 1 are 1,000 times the conductivities in reciprocal ohms obtained with the particular cell used.

In order to determine whether this method of determining the end-point was affected by the change in volume due to the addition of the reagent, a trial calculation was made with the data of Experiment 8, in which a total of 7.5 cc. of acid was added to 100 cc. of gelatin solution. Assuming as an extreme case that the conductivity was inversely proportional to the volume, and that the quantity measured

[5] Hitchcock, D. I., *J. Gen. Physiol.*, 1921–22, iv, 733.

should have been the conductivity at a constant volume of 100 cc., a curve was constructed which indicated an end-point at 4.12 cc., while that obtained by neglecting the volume change was 4.08 cc. Therefore the change in volume could not have caused an error of much over 1 per cent.

While the results in Table I do not all agree within this error, yet the average of the figures for HCl, 8.59, is quite close to the average for H_2SO_4, 8.56. Roughly, then, the data indicate that 1 gm. of isoelectric gelatin can combine with a maximum of 8.6 cc. of N/10 HCl or H_2SO_4. The figure obtained from hydrogen electrode measurements alone, corrected for an initial pH of 4.70, was 8.9 cc. of N/10 HCl.[6]

A better check was obtained in the case of deaminized gelatin. The solution used was that described in a previous paper;[6] its pH was 4.0. A 25 cc. sample, containing 0.742 gm., was titrated with 1.000 N HCl, giving an end-point at 0.323 cc. Therefore 1 gm. was equivalent to 4.35 cc. N/10 HCl, no correction for the isoelectric point being required. The value obtained by the hydrogen electrode method was 4.4 cc.

SUMMARY.

Titrations have been made, by the conductivity method, of gelatin solutions with hydrochloric and sulphuric acids. The results indicate an end-point at about 8.6 cc. of N/10 acid per gm. of gelatin, or a combining weight of about 1,160. These results are in fair agreement with those previously obtained by the hydrogen electrode method. Better agreement between the two methods was found in the case of deaminized gelatin. The data are in accord with a purely chemical conception of the combination between protein and acid.

[6] Hitchcock, D. I., *J. Gen. Physiol.*, 1923–24, vi, 95.

THEORY OF REGENERATION BASED ON MASS ACTION. II.

By JACQUES LOEB.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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I.

The polarity in the regeneration of an isolated piece of stem of *Bryophyllum calycinum* expresses itself by two characteristics which must be treated separately; namely, first, the fact that regeneration occurs only at the extreme ends, and, second, that the character of the regenerated organs is different at the opposite ends, shoots being formed at the most apical node and roots at the extreme basal end of the piece.[1] The second fact had been explained by Sachs on the assumption that the descending sap sent out by the leaf contains specific substances fit for root formation while the ascending sap sent out by the leaf contains specific substances for shoot formation. This explanation does not seem tenable any longer in view of the fact, first, that the sap of the leaf favors root and shoot formation in the same notch of a leaf, and second, that it can be shown that under proper conditions the descending sap favors also shoot formation in the stem.[2] The difference in the character of the regenerated organs at the opposite ends of a piece of stem must therefore have a different reason and it was suggested that the descending sap reaches primarily cells or tissues which can give rise to roots and not to shoots, while the ascending sap primarily reaches tissues which can give rise to shoots.[1] Differences in the chemical nature of the descending and ascending sap sent out by the leaf may or may not exist; if they exist they cannot be the cause of the different character of the organs formed at the opposite ends of the stem.

[1] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 447.
[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 831.

If by mutilation the descending sap from a leaf can be deflected from its path, it may give rise to shoots as was shown in the preceding paper.

That the regeneration in a piece of isolated stem of *Bryophyllum* is confined to the extreme ends of a piece of stem is, however, only a secondary phenomenon which demands an entirely different explanation than the fact that the character of the two regenerates is different. In a preceding paper it had already been shown that in the beginning, *i.e.*, during the first 8 or 10 days, at greenhouse temperature, the regeneration in a piece of stem does not possess that pronounced character, which is understood by polarity; namely, that roots are only formed at the extreme base and shoots only at the extreme apex. One of the writer's earlier experiments[3] may be mentioned to illustrate this fact.

Pieces of stem of *Bryophyllum calycinum* are cut out from vigorous plants and all leaves with the exception of the two leaves at the most basal node are removed from the pieces. The base of the stems below the leaves dips into water. In this case, air roots grow out from the nodes above the leaves before they grow from the base of the stem; and shoot formation commences not only in the most apical buds but also in the buds in the second or third node below the apex. Under these conditions there is almost no indication that the character of regeneration in the stem is polar.[3]

At greenhouse temperature this may all be observed within the first 8 or 10 days of the experiment, then the picture changes. At the base of the stems (dipping into water) roots now commence to appear and they grow more rapidly than the air roots which had appeared previously in the nodes of the stem. The air roots in the more apical nodes now begin to wilt and soon disappear. The more rapid growth of the basal roots dipping into water seems to suppress the further growth of the air roots which had previously formed. Likewise the shoots in the most apical node now commence to grow more rapidly than the anlagen for shoots in the nodes below the apex and the growth of these latter shoots now stops also. That feature of polarity which consists in the restriction of root formation to the extreme base and of shoot formation to the extreme apex of the piece of stem is not

[3] Loeb. I. J. *Gen. Physiol.*, 1918–19, i, 687.

a primary but a secondary phenomenon. We will show that this same phenomenon can be demonstrated in a leaf and that it can be shown in this case that it is the expression of the following general rule in regeneration which the writer had already expressed in earlier publications; namely, that when in an isolated leaf (or in a piece of stem) the rate of growth of one type of organs is accelerated, all the sap will flow to these more rapidly growing organs, with the result that the growth of the competing organs will be suppressed. It is intended to furnish the quantitative proof for this rule.

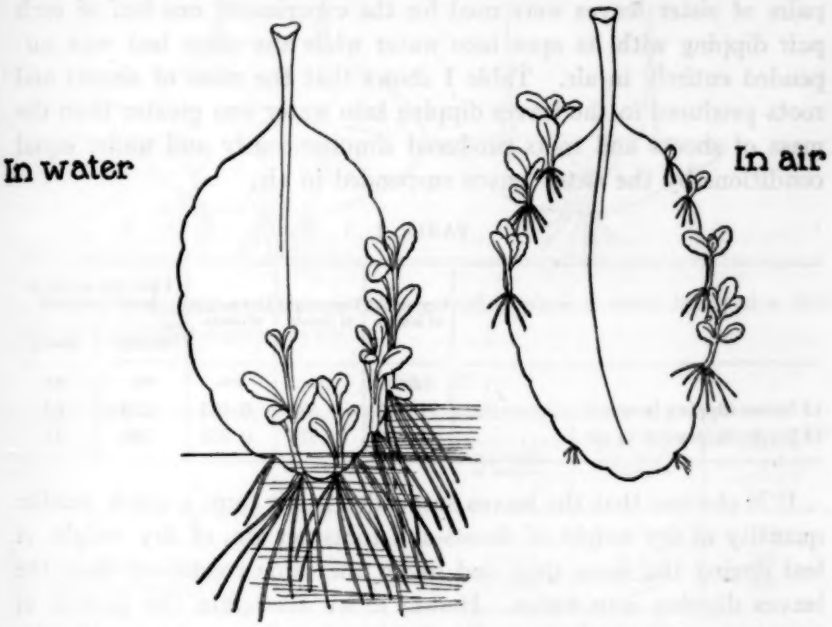

FIG. 1. Difference in the place of regeneration when the leaf is suspended entirely in air and when its apex dips into water.

When a leaf of *Bryophyllum* is suspended in moist air, shoots and roots will grow from the notches in the middle of the leaf or nearer the petiole where the leaf is more fleshy and where more sap is available than at the thin apex of the leaf (see Fig. 1, leaf marked "in air"). When, however, the apex of the leaf is dipped into water, while the rest is in air (Fig. 1, leaf marked "in water"), roots and shoots will grow out only from those notches of the apex which dip into the water

or are close to the surface of the water. The other notches commence also to grow at first and tiny roots and tiny shoots may be formed; but as soon as the notches which dip into water commence to grow, all the growth of the shoots and roots in the other notches not in contact or in close proximity with water will cease. It can be shown that the reason for this inhibition is that the notches dipping into water grow more rapidly than the notches growing in air and that as a consequence of this more rapid growth a flow of the sap of the whole leaf towards the more rapidly growing notches is established. Thirteen pairs of sister leaves were used for the experiment, one leaf of each pair dipping with its apex into water while the sister leaf was suspended entirely in air. Table I shows that the mass of shoots and roots produced in the leaves dipping into water was greater than the mass of shoots and roots produced simultaneously and under equal conditions by the sister leaves suspended in air.

TABLE I.

	Dry weight of leaves.	Dry weight of shoots.	Dry weight of roots.	1 gm. dry weight of leaves produced	
				Shoots.	Roots.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*
13 leaves dipping in water.....	1.943	0.524	0.123	270	63
13 leaves suspended in air.....	1.909	0.322	0.051	169	27

It is obvious that the leaves suspended in air form a much smaller quantity of dry weight of shoots and roots per gm. of dry weight of leaf during the same time and under the same conditions than the leaves dipping into water. Hence, if we accelerate the growth of some notches in the leaf, *e.g.*, by dipping them into water, we thereby inhibit the growth in the other notches.

That this inhibition is merely due to the fact that all the material available in the leaf now goes to the shoots in the apex can also be proven by quantitative experiments. The writer had shown in a previous publication[3] that if an isolated leaf is suspended vertically and sidewise in moist air (Fig. 2), *i.e.*, the middle rib being in a horizontal position, while the surface of the leaf is vertical (Fig. 2), shoots and roots develop with preference, and in most cases exclusively, on

the lower edge of the leaf. This can be explained on the assumption that through the influence of gravity more liquid collects in the lower edge and that as a consequence the growth in these lower notches is accelerated, and as a further consequence a flow of material to the more rapidly growing lower notches is established, whereby all the material available in the leaf flows to the lower edge.

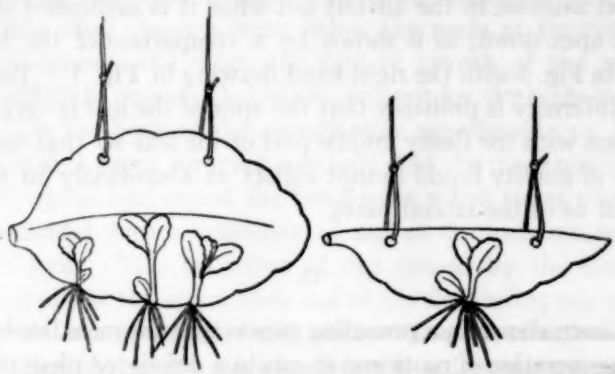

FIG. 2. Quantitative difference in regeneration when a whole leaf and a half leaf are suspended sidewise in air.

TABLE II.

	Dry weight of leaves.	Dry weight of shoots.	1 gm. dry weight of leaves produced dry weight of shoots.
	gm.	*gm.*	*mg.*
13 whole leaves	2.174	0.410	188
13 half sister leaves	1.118	0.235	209

A number of pairs of sister leaves were suspended vertically and sidewise in air, one leaf of each pair remaining intact, while the upper half of the sister leaf was cut away (as shown in Fig. 2). The whole leaves formed practically twice as great a mass of shoots and roots on the lower edge as the half leaves and this difference is noticeable in Fig. 2. Thirteen whole leaves and their thirteen half sister leaves were chosen for comparison in Table II.

This proves that the excess regeneration in the ***whole*** leaves was produced by material furnished by both the lower and the upper half

of the leaves, regeneration in the upper half being as a consequence impossible, since almost all the material available for regeneration in the upper half was consumed for regeneration in the lower half.

In the case just discussed the regeneration in the lower half of the leaf is accelerated since owing to the action of gravity the liquid in the leaf collects in the lower half. This happens only when the leaf is suspended sidewise in the air but not when it is suspended in the air with the apex down; as is shown by a comparison of the left-hand drawing in Fig. 2 with the right-hand drawing in Fig. 1. The reason for this difference is probably that the apex of the leaf is very thin in comparison with the fleshy middle part of the leaf so that under the influence of gravity liquid cannot collect as abundantly in the apex of the leaf as in the lateral parts.

II.

It had been shown in a preceding paper[1] that the mass law holds also for the regeneration of roots and shoots in a defoliated piece of stem of *Bryophyllum* when exposed to light. The dry weight of shoots and roots regenerated by such pieces in a given time under given conditions is approximately in proportion to the dry weight of the stem; and when a long stem of *e.g.* eight nodes is cut into eight pieces, each containing only one node, the total dry weight of the sixteen shoots produced by these pieces in a given time equals approximately the dry weight of the two apical shoots produced in the same time and under the same conditions by a stem of equal dry weight, not cut into smaller pieces. This shows that all the material available for regeneration in a larger piece of defoliated stem goes into the apical shoots and basal roots.

When a piece of stem is cut into as many pieces as there are nodes the shoots commence to grow out from the nodes regardless of the order in which they had originally been arranged in the stem; but in each piece the rate of growth corresponds approximately to the dry weight of the piece of stem, except in very old pieces of stem where not all material may be alive or in the very young pieces at the apex, where the axillary buds capable of growing into shoots may not yet have been properly developed.[1]

In the introduction to this note attention has been called to the fact that when regeneration begins in a piece of stem under the conditions described, regeneration is at first not markedly polar, since the first roots do not grow out at the base of the stem, but from the more apical nodes; and the anlagen for shoots do not grow out only in the apical node, but also below. The polar character becomes only established after about a week, when the roots at the extreme base begin to appear, and when the rate of growth of the most apical shoots suddenly exceeds that of the anlagen for the buds in the lower nodes. On the basis of the quantitative experiments on leaves described in this paper we must conclude that the more rapid growth of the most apical bud and of the basal roots which starts after about a week coincides with a collection of sap at the extreme ends of the piece of stem. This collection of sap caused by the block of the ascending sap at the apical node and of the descending sap at the base of the stem acts in a similar way as the collection of sap on the lower edge of a leaf suspended sidewise; namely, accelerating growth at the place where the sap collects. This acceleration of growth now influences the sap flow so that all the ascending sap goes to the apical node, and all the descending sap to the base, cutting off that supply of material for the rest of the stem which was available immediately after the operation.

SUMMARY.

1. Quantitative proof is furnished that all the material available for shoot and root formation in an isolated leaf of *Bryophylium calycinum* flows to those notches where through the influence of gravity or by a more abundant supply of water growth is accelerated. As soon as the acceleration of growth in these notches commences, the growth of shoots and roots in the other notches which may already have started ceases.

2. It had been shown in a preceding paper that the regeneration of an isolated piece of stem may be and frequently is in the beginning not markedly polar, but that after some time the growth of all the roots except those at the base and of all the shoots except those at the apex is suppressed. This analogy with the behavior of regenera-

tion in a leaf in which the growth in one set of notches is accelerated, suggests that in an isolated stem a more rapid growth is favored at the extreme ends (probably by a block of the sap flow at the extreme ends) and that when this happens the total flow of ascending sap goes to the most apical buds and the total flow of the descending sap goes to the most basal roots. As soon as this occurs, the growth of the other roots and shoots is suppressed.

THE INFLUENCE OF THE CHEMICAL NATURE OF SOLID PARTICLES ON THEIR CATAPHORETIC P.D. IN AQUEOUS SOLUTIONS.

By JACQUES LOEB.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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I.

INTRODUCTION.

In order to arrive at a theory of the action of electrolytes on the formation of a double electrical layer—or, more correctly, on the value of the cataphoretic P.D.—between solid particles and aqueous solutions, it is necessary to know how this action is influenced by the nature of the suspended particle. In former papers by the writer, the effect of electrolytes on the cataphoretic P.D. was studied on proteins[1] and on collodion particles.[2] In this paper experiments on particles of mastic, Acheson's graphite (trade name "Aquadag," Size 5), which I owe to the kindness of Dr. Leonard Waldo, gold (prepared by Bredig's method in solution of N/10,000 HCl), ferric hydroxide, and calcium oxalate are added. To give a more complete picture experiments on collodion and protein are included.

In order to eliminate as much as possible the influence of H and OH ions, the experiments with salts were made in distilled water as near the point of neutrality as was conveniently possible. The distilled water used had a pH of about 5.8, resulting from an equilibrium with the CO_2 of the air of the laboratory. Stock solutions of the salts were prepared with a pH of 5.8 and these were diluted with distilled water of the same pH. Great care was taken to keep the pH of the solutions at 5.8, except in the case of solutions of $ThCl_4$ which were always more acid on account of the hydrolytic

[1] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 395.

[2] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 109; 1923-24, vi, 105.

dissociation of the salt; and of solutions of acid and basic fuchsin, where the pH was not measured.

It was also thought advisable to eliminate impurities as much as possible. Such impurities were contained in some cases in the stock solution of the suspended particles. This error was minimized by using as small a quantity of suspended particles as possible. A few drops of the stock suspension were put into 50 cc. of the salt solution, shaken, and allowed to stand for 20 minutes. When this is done, it is found that the P.D. of the particles in distilled water of pH 5.8 without the presence of salts is generally very small, rarely above 8 millivolts. Only when the particles are themselves electrolytes or when electrolytes are added does the P.D. rise. Statements in the literature that a high cataphoretic P.D. was observed in distilled water without electrolytes suggest the presence of electrolytes in the form of impurities, unless the particle itself was an electrolyte. The value of 26 millivolts for mastic particles in pure water at pH 5.8 in Fig. 2 is too high and unquestionably due to an impurity of some kind. A repetition of the experiments where such errors were excluded gave a much lower P.D., namely about 8 millivolts or less.

Although the P.D. of the particles in water at pH 5.8 is very low in the absence of electrolytes, the sign of charge of the particles at this low P.D. is of the greatest significance for the effect of the salt on the P.D. This effect is entirely different according to whether a particle is positively or negatively charged in pure water near the point of neutrality even if the P.D. be as low as 5 millivolts. We shall therefore designate as negative colloids, such particles which at pH 5.8 are negatively charged in the absence of electrolytes; and as positive colloids, particles which are positively charged at pH 5.8 in the absence of electrolytes.

The method of determining the cataphoretic P.D. was the same as in the preceding papers, namely, the measurement of the rate of migration of isolated particles under the microscope in the way described by Ellis.[3] From the measurements of the velocity of migration the P.D. was calculated with the aid of the Helmholtz-Perrin formula. The apparatus used was that by Ellis as modified by Northrop,[4] with

[3] Ellis, R., *Z. physik. Chem.*, 1911–12, lxxviii, 321; 1912, lxxx, 597.

[4] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 629.

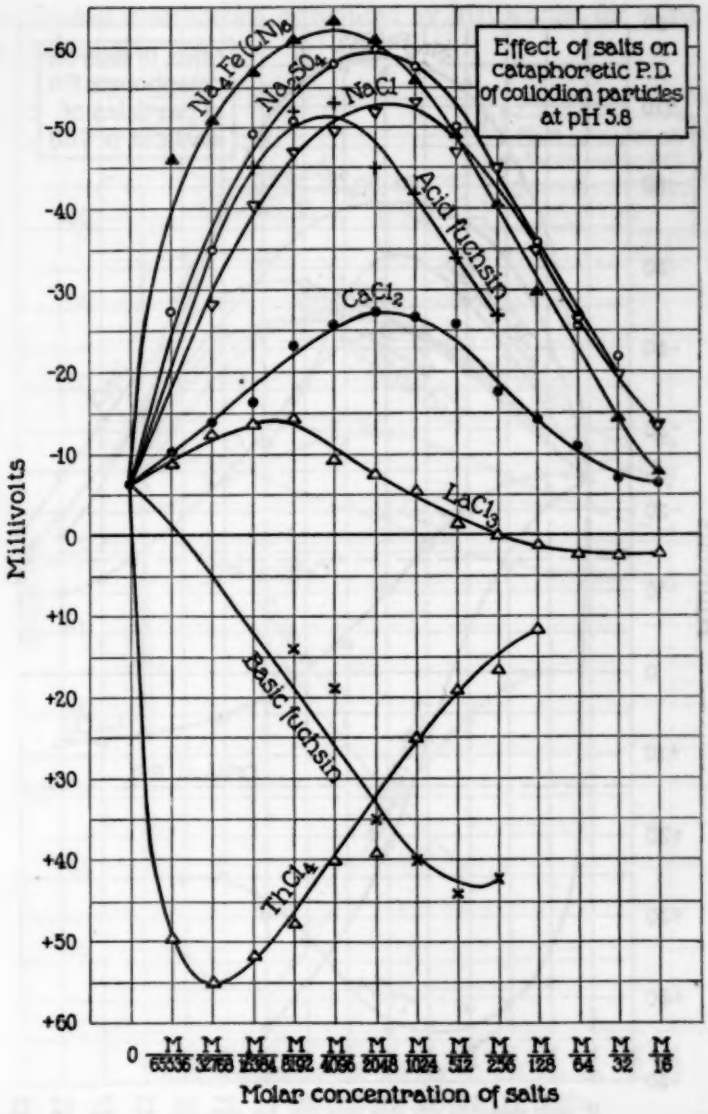

FIG. 1. Influence of salts on the cataphoretic P.D. between collodion particles and aqueous solutions near the point of neutrality, pH 5.8, (with the exception of the solutions of $ThCl_4$ and basic fuchsin). Abscissæ are the molar concentrations of salt solutions, ordinates, the cataphoretic P.D. The signs minus and plus refer to the sign of charge of the particles.

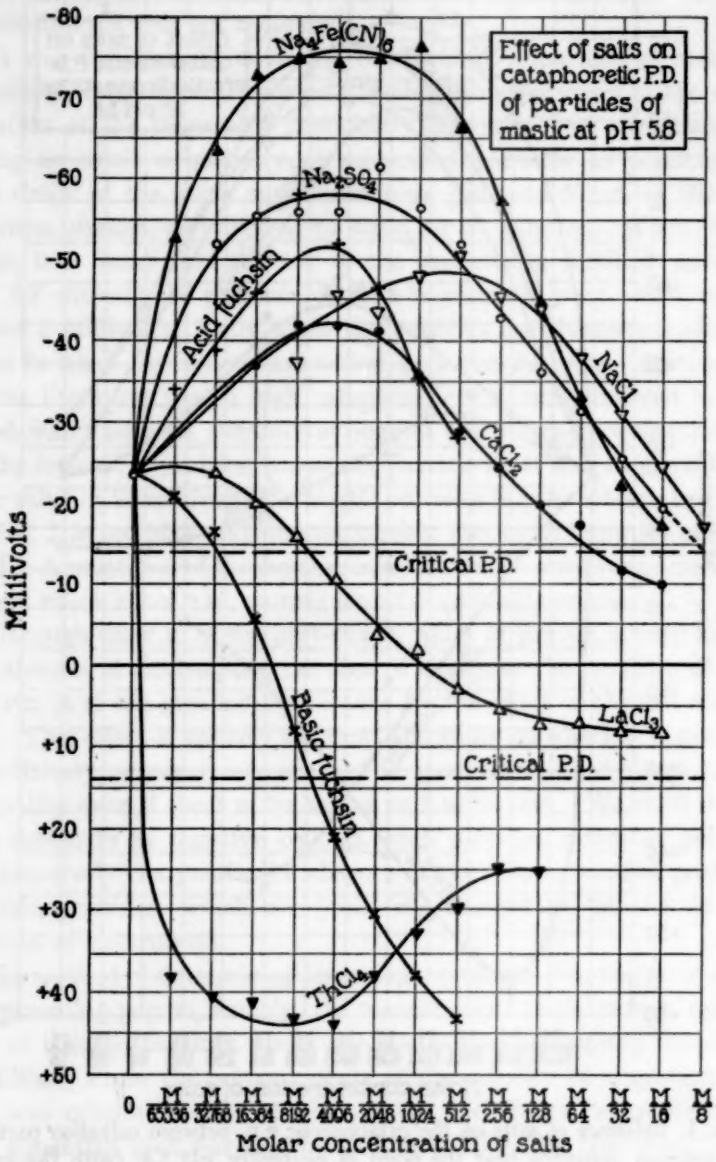

FIG. 2. Influence of salts on the cataphoretic P.D. of mastic particles near neutrality (pH 5.8). The line Critical P.D. indicates the value of P.D. below which the suspension ceases to be stable.

an additional modification introduced by Mr. Kunitz for using dark-field illumination.

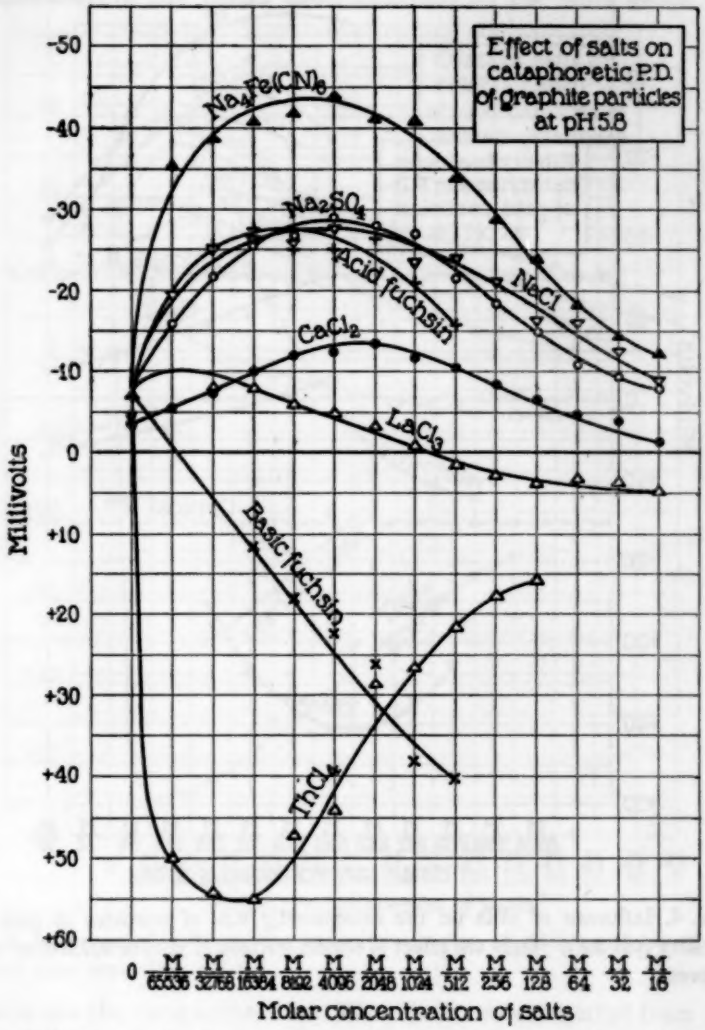

FIG. 3. Influence of salts on the cataphoretic P.D. of particles of Acheson's graphite (trade mark "Aquadag," Size 5) near neutrality, pH 5.8.

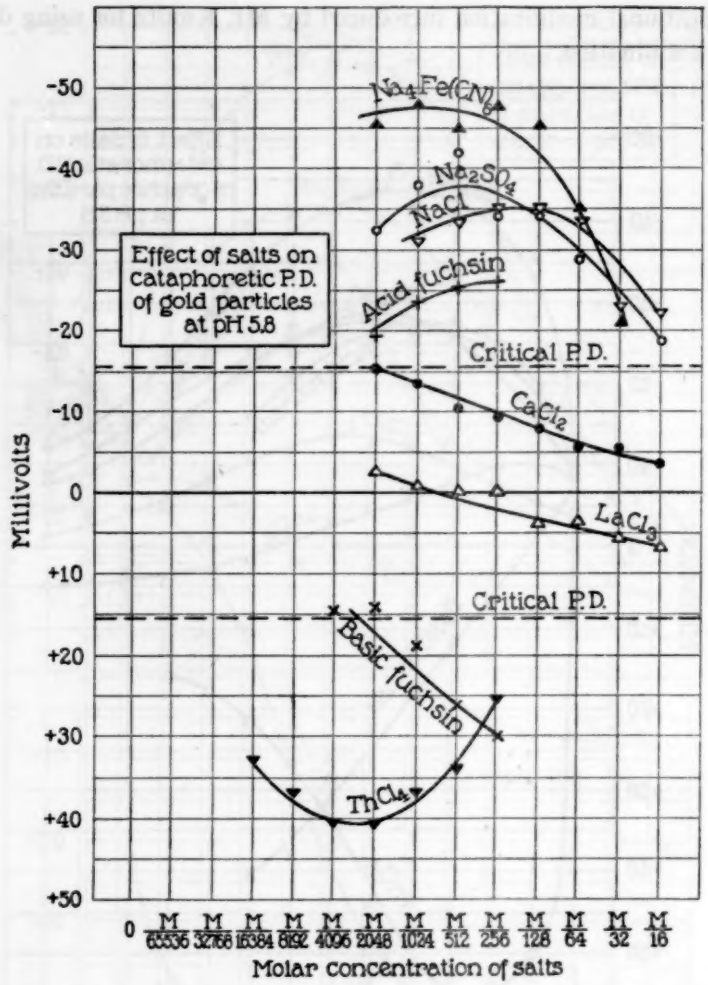

FIG. 4. Influence of salts on the cataphoretic P.D. of particles of gold near neutrality (pH 5.8). Only the effect of concentrations of salts of M/2,048 or above are given.

II.

Experiments on Negatively Charged Particles.

Six types of particles, negatively charged at pH 5.8, were selected for this study, collodion (Fig. 1), mastic (Fig. 2), Acheson's graphite

(Fig. 3), gold (Fig. 4), Na gelatinate (Fig. 5), and crystals of tyrosine. (At pH 5.8 the tyrosine is little if at all dissociated.) In Figs. 1 to 5 the abscissæ are the molar concentrations of the salts while the

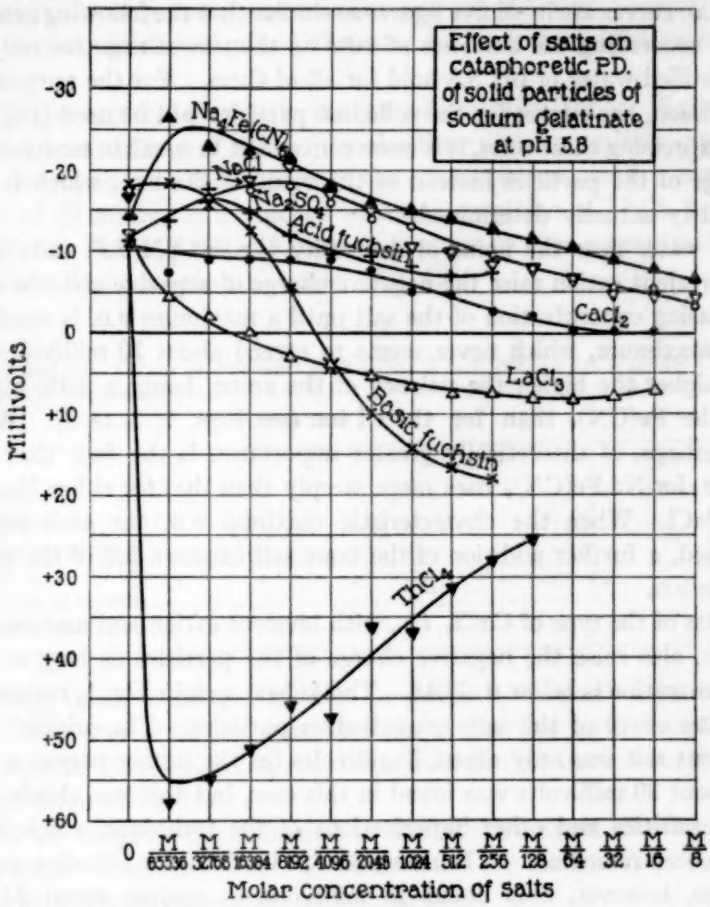

FIG. 5. Influence of salts on the cataphoretic P.D. of solid particles of Na gelatinate near neutrality, pH 5.8.

ordinates are the cataphoretic P.D. in millivolts as calculated from the rate of migration with the aid of the Helmholtz-Perrin formula.[5]

[5] Freundlich, H., Kapillarchemie, Leipsic, 2nd edition, 1922, 326 ff. Burton, E. F., The physical properties of colloidal solutions, London, New York, Bombay, Calcutta, and Madras, 2nd edition, 1921, 136 ff.

The sign of the charge is always that of the particle, the negative charges being above the zero line, the positive charges below. In spite of the wide divergence in the chemical nature of the material, the P.D. curves are in all five figures so similar that the following general rules concerning the influence of salts on the P.D. of negative colloids in distilled water of pH 5.8 hold for all of them. For the purpose of discussion, the curves for the collodion particles will be used (Fig. 1). (In expressing these rules, it is more convenient to speak in terms of the charge of the particles instead of the value of the P.D., which is the quantity actually determined.)

In water near the point of neutrality (*i.e.*, at pH 5.8) salts with monovalent cation raise the negative charge of negative colloids with increasing concentration of the salt until a maximum P.D. is reached; this maximum, which never seems to exceed about 70 millivolts,[6] is the higher the higher the valency of the anion, being a little higher for the $Fe(CN)_6$ than for the Cl ion (see Figs. 1, 2, or 3). What is, perhaps, of theoretically greater importance is the fact that the curve for $Na_4Fe(CN)_6$ rises more steeply than that for either Na_2SO_4 or NaCl. When the characteristic maximal P.D. for each salt is reached, a further addition of the same salt causes a fall of the value of the P.D.

Salts of the type of $CaCl_2$, *i.e.*, with bivalent cation and monovalent anion, also raise the negative charge of the particles as long as the concentration is below M/2,048. This is best seen in Fig. 1, representing the effect of the salts on collodion particles. The original P.D. without salt was only about 7 millivolts (in the former papers a P.D. of about 30 millivolts was found in this case, but this was chiefly due to impurities and other imperfections of the technique which have since been remedied). The maximal P.D. in $CaCl_2$ for collodion particles is, however, only about 28 millivolts as against about 53 for NaCl (Fig. 1).

Even a salt like $LaCl_3$ still raises the P.D. (Fig. 1), but only from 7 to about 15 millivolts. The maximal P.D. is reached in $LaCl_3$ at a molar concentration of between M/16,000 and M/8,000. With the addition of more $LaCl_3$ the P.D. is depressed. At a concentration of

[6] von Hevesy, G., *Kolloid Z.*, 1917, xxi, 129. Lorenz, R., Raumerfüllung und Ionenbeweglichkeit, Leipsic, 1922, 237.

about M/128 the particles become slightly positive, but the P.D. remains at nearly zero even in M/16 $LaCl_3$. In the writer's former experiments the fact that $LaCl_3$ is able to raise the P.D. of collodion particles in very low concentrations was concealed on account of the impurities which had raised the P.D., without the additions of any salt.

No rise occurs, however, in the case of a salt like $ThCl_4$. Extremely low concentrations of this salt make the particles positive, and the P.D. increases with the increase of the concentration, until a maximal P.D. of 55 millivolts is reached at a concentration of $ThCl_4$ between M/30,000 and M/8,000. A further increase in the concentration of the salt depresses the P.D. again (Fig. 1).

TABLE I.

Maximal P.D. *Values in Solutions of* $Na_4Fe(CN)_6$ *at pH 5.8 for Negative Colloids.*

Nature of particles.	P.D.
	millivolts
Mastic	about 75
Collodion	" 62
Acheson's graphite	" 43
Gold	" 47
Crystals of tyrosine	" 45
Na gelatinate	" 25

High positive charges are also produced by basic fuchsin and the P.D. increases with the concentration of the dye until a maximum is reached after which the P.D. drops with further increase in the concentration of the dye.

If we compare the difference in the relative effect of salts on the six negative colloids (Table I) we may use the maximal P.D. values in $Na_4Fe(CN)_6$ as a standard of comparison.

The values for Na_2SO_4 and NaCl vary correspondingly.

III.

Positively Charged Particles.

Figs. 6, 7, and 8 show the effects of salts on positively charged particles; namely, ferric hydroxide, calcium oxalate, and casein

chloride. The experiments with ferric hydroxide and calcium oxalate were made at pH 5.8 and care was taken that this pH was not altered by the addition of the salts (with the exception of the dyes). The experiments with casein chloride had to be made on the acid side of the isoelectric point of casein and the pH of 4.0 was selected. The reader

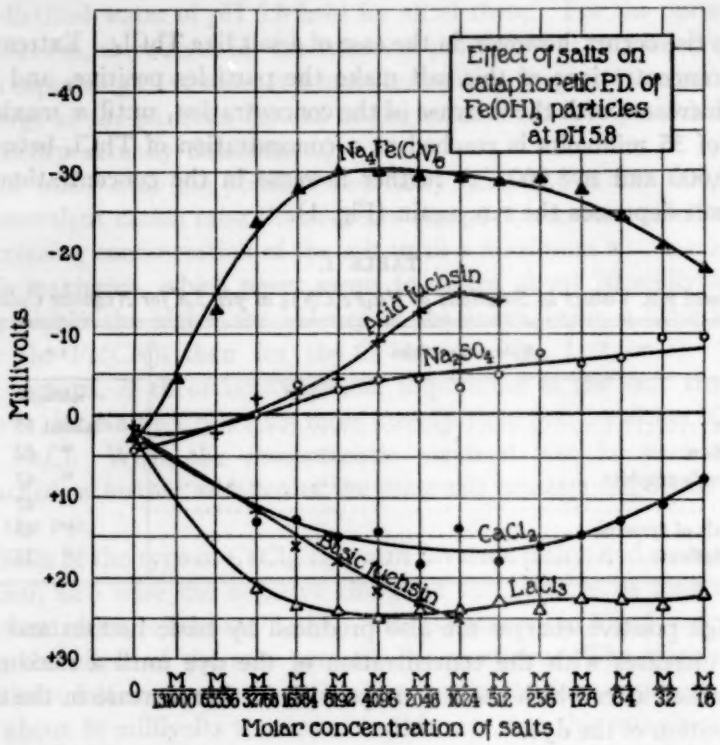

FIG. 6. Influence of salts on the cataphoretic P.D. of particles of $Fe(OH)_3$ near neutrality, pH 5.8.

will notice that all three positively charged particles are electrolytes while in the case of the negatively charged particles some, *e.g.* Na gelatinate, were electrolytes, while collodion and mastic were apparently non-electrolytes.

The curves for the positive colloids differ in an essential respect from those for negative colloids. While in the case of negative colloids the Cl and SO_4 ions had a powerful augmenting effect on the P.D.

these two anions have almost no effect on the positive colloids. Na_2SO_4 makes the positive colloids slightly negative at comparatively high concentrations but the P.D. is very small indeed. Only $Na_4Fe(CN)_6$ and acid dyes make the positive particles negative in rather low molar concentrations, but even here the P.D. remains low (30 millivolts or less).

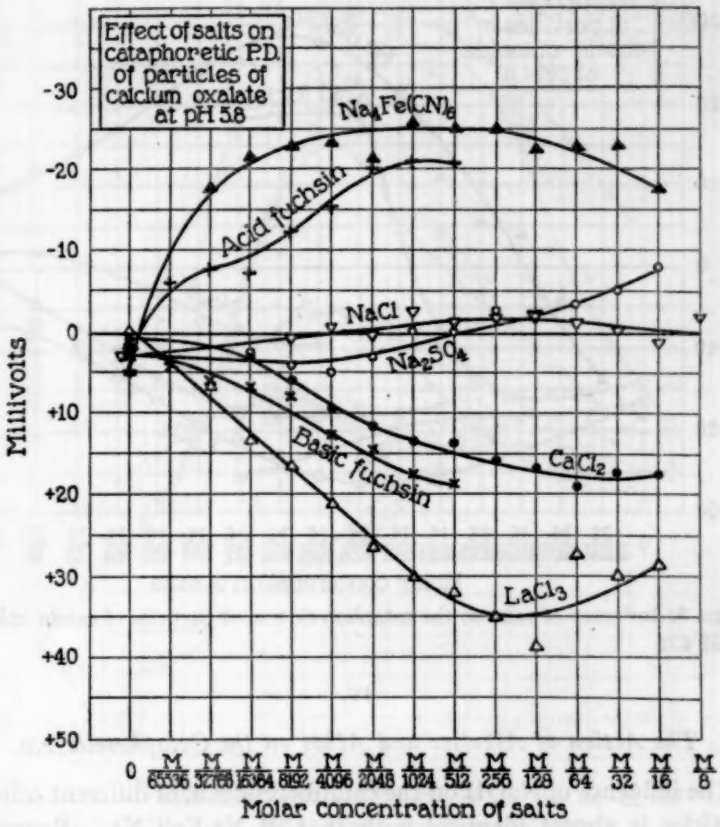

FIG. 7. Influence of salts on the cataphoretic P.D. of particles of calcium oxalate near neutrality, pH 5.8.

Perhaps, it is on account of this lack of efficiency of the Cl ion, that $CaCl_2$ and $LaCl_3$ in low concentrations increase the positive charge of the positive colloids until a maximal P.D. is reached after which a

further increase in the concentrations of $CaCl_2$ and $LaCl_3$ will depress the P.D. again.

Basic fuchsin does not act quite as strongly as does $LaCl_3$ on the P.D. of positive colloids but in the same sense.

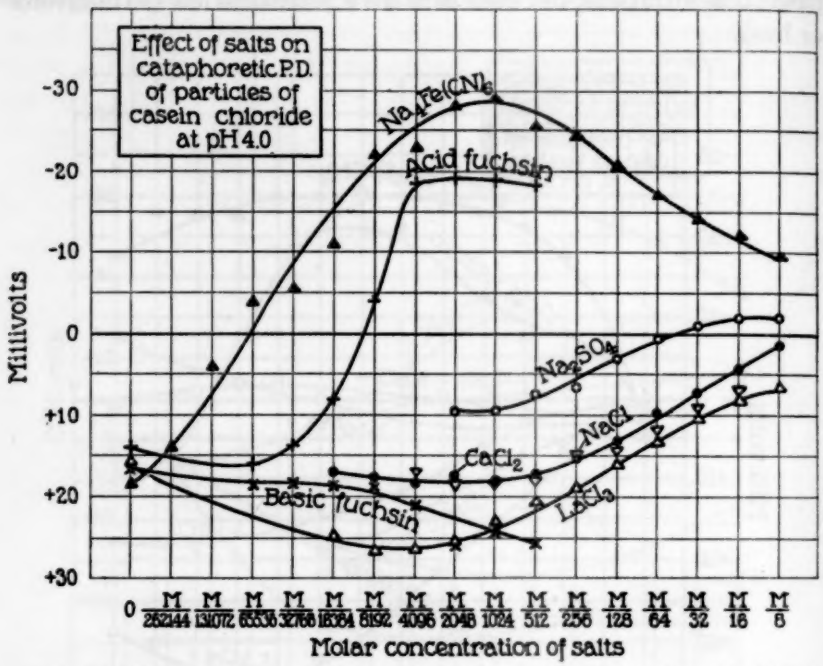

FIG. 8. Influence of salts on the cataphoretic P.D. of particles of casein chloride at pH 4.0.

IV.

The Action of Alkalies and Acids on the Cataphoretic P.D.

The influence of NaOH on the cataphoretic P.D. of different colloidal particles is almost identical with that of $Na_4Fe(CN)_6$. Regardless of whether the colloid is originally negative or positive, NaOH makes the particles always more negative and the P.D. increases until a maximum is reached at about M/4,096 or M/1,024; after which the P.D. drops again upon further increase in the concentration of the alkali (Fig. 9). The OH ion is therefore driven with about the

same relative force into the enveloping film as is the $Fe(CN)_6$ ion. Table II gives the maximal values of the P.D. in NaOH solutions.

It might be inferred *a priori* that hydrogen ions act like thorium ions and this would seem to receive support in the statement so often

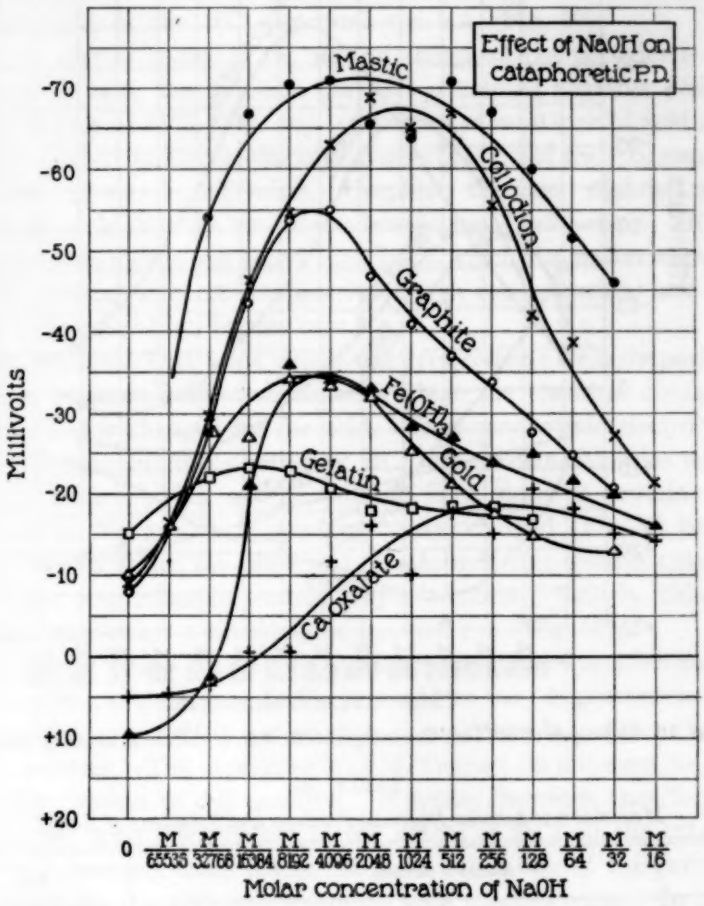

FIG. 9. Influence of NaOH on the cataphoretic P.D. of different kinds of particles.

found in the colloidal literature that hydrogen ions make colloids positive. While this is true for amphoteric electrolytes, Fig. 10 shows that this cannot be generalized. Low concentrations of HCl

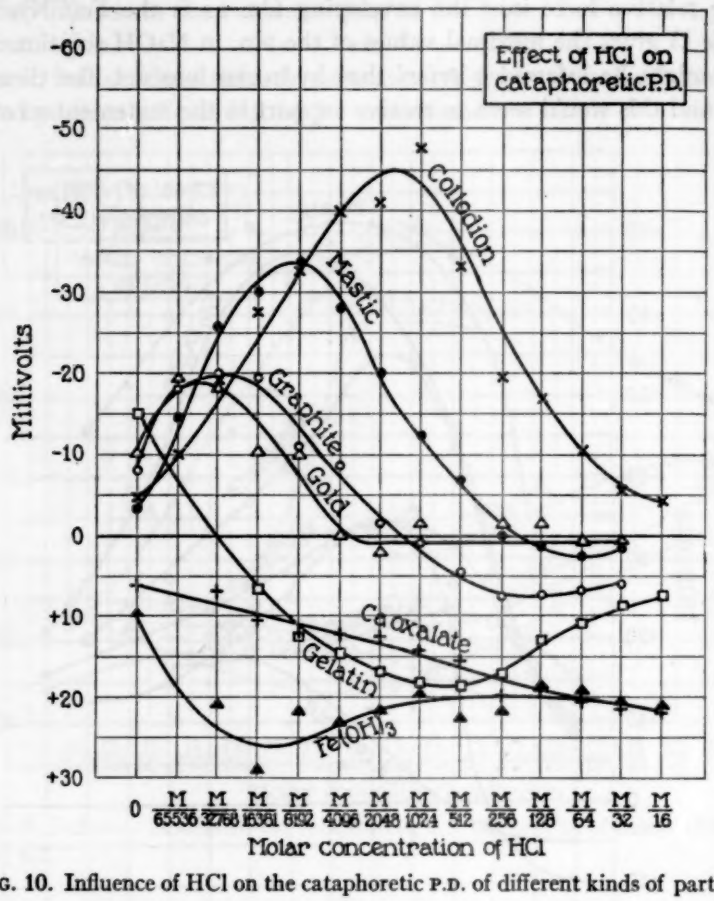

FIG. 10. Influence of HCl on the cataphoretic P.D. of different kinds of particles.

TABLE II.

Maximal P.D. *between Different Particles and Solutions of NaOH.*

Nature of particles.	P.D.
	millivolts
Mastic	71
Collodion	67
Acheson's graphite	55
Gold	50
$Fe(OH)_3$	35
Na gelatinate	23
Ca oxalate	18

generally increase the negative charge of negative colloids such as collodion, mastic, Acheson's graphite, and gold (Fig. 10) until a maximum is reached, which is quite high in the case of collodion (about 45 millivolts), lower in the case of mastic (about 33 millivolts), graphite, and gold (about 20 millivolts). A further increase in the concentration of the HCl depresses the P.D. (Fig. 10).

In the case of collodion the sign of charge of the particles is not reversed by acid, the collodion particles remaining negative even in M/8 solutions of HCl (Fig. 10). Particles of mastic or of gold may become positive in higher concentrations of HCl, but the P.D. remains very low (2 or 3 millivolts). Graphite becomes slightly more positive in acid, but the P.D. is also low (about 7 millivolts). HCl is therefore even a little less active than $LaCl_3$ which also makes negative colloids positive only at comparatively high concentrations and the P.D. is also never high in this case.

The statement that acids render negative colloids strongly positive seems to be correct only in such cases where the chemical character of the colloid is changed by the acid. Thus amphoteric electrolytes like the proteins are transformed by HCl into protein chlorides where the non-diffusible ion of the particle is a positive protein ion. Particles of Na gelatinate are negatively charged (Fig. 4) but if enough HCl is added the protein is transformed into gelatin chloride where the non-diffusible protein ion is a cation. Gelatin chloride particles may reach a considerable positive P.D. (Fig. 10).

McTaggart[7] states that $Th(NO_3)_4$ makes gas bubbles positive and so does also to a slight extent $LaCl_3$; it would be very important to find out whether or not HCl can produce a considerable positive charge on gas bubbles. The experiments of McTaggart do not seem to give a definite answer to this question. It seems, therefore, that for the present we must discriminate in the effects of HCl on the cataphoretic P.D. between cases where the chemical nature of the particles is changed by the acid and where no such changes occur. In cases of the latter type (presumably collodion particles), solutions of

[7] McTaggart, H. A., *Phil. Mag., Series 6*, 1914, xxvii, 297; xxviii, 367; 1922, **xliv**, 386.

NaOH and HCl may act like neutral solutions of NaCl on the cataphoretic P.D. as is shown in Fig. 11, the only difference between the three electrolytes being that the OH ion acts a little more strongly than the Cl ion and the H ion slightly more strongly than the Na ion;

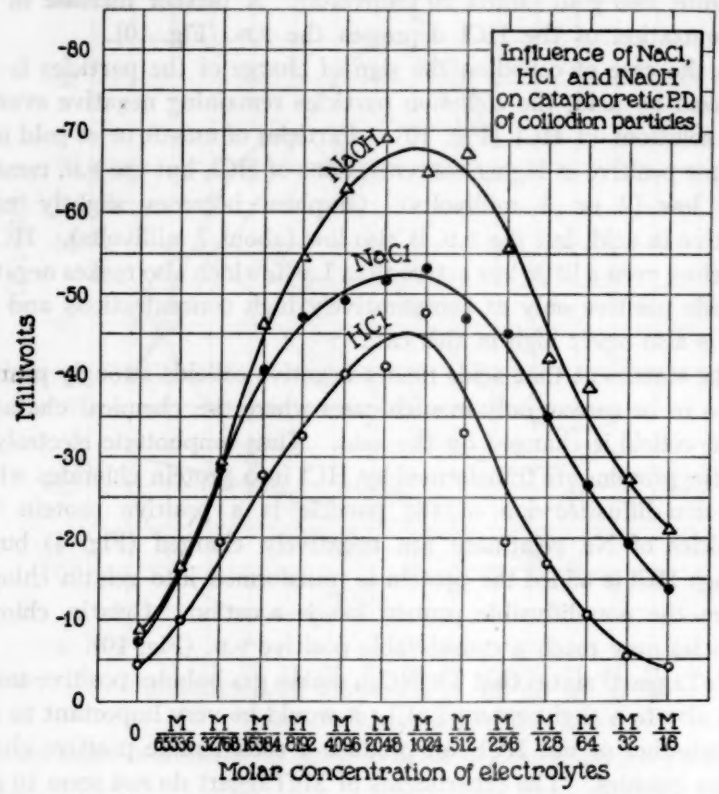

FIG. 11. Comparison of the influence of HCl, NaCl, and NaOH on the cataphoretic P.D. of collodion particles. The curve for NaCl represents the effects of this salt at an approximately neutral reaction, pH 5.8.

or, if we use the suggestion of a relative orientation of the ions, along the normal to the interface, OH ions are driven with a slightly greater force into the enveloping film than the Cl ions, and the H ions are driven with a slightly smaller force away from the interface than the Na ions.

V.

Flocculation.

Although it is not an essential part of our problem it may perhaps be of importance to point out that there seems to exist in the case of all of these suspensions a critical P.D. below which they cease to be stable and commence to flocculate. It is rather remarkable that this critical P.D. differs very little with the chemical nature of the suspended particles. The critical P.D. is indicated by lines marked Critical P.D. in Figs. 2 and 4. The suspensions of collodion,[2] mastic, graphite, and gold cease to be stable and flocculation commences when the cataphoretic P.D. falls to a value of between 16 and 13 millivolts. Northrop and De Kruif[8] found a similar value, namely 15 millivolts, for the flocculation of certain bacteria. The values for the flocculation of suspensions of calcium oxalate, denatured casein, and denatured albumin[9] are not very far from this value. The interpretation of this fact seems to be that flocculation can only occur when the average velocity of the particles is high enough to overcome the repelling force due to the P.D. of the double layer. The average velocity of the particles seems to be such that it suffices to overcome the repulsion due to a P.D. of about 14 millivolts or less. Flocculation requires, however, something more than the mere overcoming of the repulsive forces due to the cataphoretic P.D.; namely, the force of cohesion between the particles themselves must be considerably greater than the forces of adhesion between the particles and the water. Particles of gelatin (or of collodion coated with gelatin) will not flocculate even if the P.D. is zero, and the same is true for particles of denatured egg albumin or casein in the presence of gram molecular concentrations of NaCl or $CaCl_2$.[9] In these cases the forces of cohesion of the particles are relatively small compared with the attraction of the particles for water, so that they fail to cohere upon collision even if the cataphoretic P.D. is zero.

It has frequently been stated that the precipitating power of electrolytes increases with the mobility of the active ion.[10] Thus the

[8] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639.

[9] Loeb, J., *J. Gen. Physiol.*, 1922–23, v, 479.

[10] Mukherjee, J. N., *Farad. Soc. Rep.*, London, 1921, xx, 103.

precipitating power of the chlorides of alkali metals is said to increase in the order Li < Na < K < Rb < Cs. This cannot be a general rule, since LiCl, NaCl, and KCl influence the P.D. of collodion as well as graphite particles in an identical way. Flocculation is determined by the critical P.D. and since identical concentrations of LiCl, NaCl, and KCl are required to bring about the critical P.D., the flocculating concentrations for these salts should be identical. This was found to be true for suspensions of mastic, graphite, and collodion when care was taken to keep the pH constant. In Tables III, IV, and V are given the flocculating concentrations of different salts at pH 5.8 for suspensions of mastic, Acheson's graphite, and gold. (Only in the

TABLE III.

Mastic.

	Flocculation.	Suspension.
LiCl	M/4	M/8
NaCl	M/4	M/8
KCl	M/4	M/8
RbCl	M/2	M/4
CsCl	M/4	M/8
$MgCl_2$	M/32	M/64
$CaCl_2$	M/32	M/64
$SrCl_2$	M/32	M/64
$BaCl_2$	M/32	M/64
$MnCl_2$	M/32	M/64
$LaCl_3$	M/8,192	M/16,000
$ThCl_4$	M/260,000	$\lesssim$ M/260,000
Basic fuchsin	M/8,000 – M/4,000	>M/4,000 <M/8,000

case of $ThCl_4$ and basic fuchsin were the pH different.) By flocculation is meant the complete settling of the particles, leaving a completely colorless and clear supernatant liquid; while by suspension is meant that the liquid remains red in the case of gold or gray or opaque in the case of mastic or black in the case of graphite; while no or little settling occurs. The second column of the tables gives the minimal molar concentration required for flocculation, and the third column the highest concentration at which the suspensions remain stable for weeks. In order to get unequivocal results, the values in Columns

TABLE IV.

Graphite (Aquadag).

	Flocculation.	Suspension.
LiCl	M/32	M/64
NaCl	M/32	M/64
KCl	M/32	M/64
RbCl	M/32	M/64
CsCl	M/32	M/64
$MgCl_2$	M/512	M/1,024
$CaCl_2$	M/1,024	M/2,048
$SrCl_2$	M/512	M/1,024
$BaCl_2$	M/1,024	M/2,048
$MnCl_2$	M/1,024	M/2,048
$LaCl_3$	M/65,000	M/130,000
$ThCl_4$	M/500,000	$\lesssim$ M/500,000
Na_2SO_4	M/64	M/128
$Na_4Fe(CN)_6$	M/64	M/256
$MgSO_4$	M/512	M/1,024
Basic fuchsin	M/8,000 and M/16,000	>M/8,000 <M/16,000
HCl	M/2,048	M/4,096

TABLE V.

Gold.

	Flocculation.	Suspension.
LiCl	M/16	M/32
NaCl	M/64	M/128
KCl	M/128	M/256
RbCl	M/16	M/32
CsCl	M/128	M/256
$MgCl_2$	M/16,000	M/32,000
$CaCl_2$	M/4,096	M/8,192
$SrCl_2$	M/8,192	M/16,000
$BaCl_2$	M/16,000	M/32,000
$MnCl_2$	M/8,192	M/16,000
$LaCl_3$	M/260,000	M/500,000
$ThCl_4$	M/500,000	
Na_2SO_4	M/64	M/128

2 and 3 were ascertained after the suspensions had been standing in test-tubes for a week or more at room temperature. Variations occurred only in the case of gold, but they were too irregular and could not be reproduced with any degree of certainty. The writer cannot suppress a suspicion that the variations in the flocculating concentrations of salts in the case of gold particles were the result of traces of impurities introduced accidentally. In Table V only the red suspensions were permanently stable, while those which at the beginning were blue or bluish red had settled.

Odén[11] observed enormous differences in the precipitating power of the salts of the alkali metals on suspensions of colloidal sulfur. Nothing of this kind can be observed in the case of the precipitation of mastic, Acheson's graphite, collodion particles, or even particles of gold.

VI.

Theoretical Remarks.

There are two kinds of forces which can cause an excess of one kind of ions in the enveloping film; namely, forces inherent in the water itself and forces of attraction between the colloidal particles and the ions.

The share which forces inherent in the water have in the orientation of the oppositely charged ions of an electrolyte in the interface can only be ascertained through experiments on the influence of electrolytes on the cataphoretic migration of gas bubbles in aqueous solutions, but experiments of this kind are as yet too incomplete to permit more than the statement that in cataphoresis gas bubbles are generally negatively charged, that their sign of charge is reversed by low concentrations of $Th(NO_3)_4$ and presumably by higher concentrations of $LaCl_3$; and that it is doubtful whether acids alone can cause a noticeable reversal in the sign of charge of gas bubbles (*i.e.* with a P.D. exceeding a few millivolts). If we apply these meager data to the experiments reported in this paper we may say that the effect of $ThCl_4$ or $LaCl_3$ or even of strong acids on dielectrics like collodion or

[11] Odén, S., Der kolloide Schwefel, *Nova acta regiæ Soc. Sc. Upsaliensis, Series 4*, 1913, iii, No. 4.

mastic are similar to those observed on gas bubbles and that therefore the effects of these three groups of electrolytes on the cataphoretic P.D. of dielectrics may be partly or entirely due to forces inherent in the water itself. Correspondingly it is quite possible that the effect of $Na_4Fe(CN)_6$ which reverses the sign of charge of positive particles may turn out to be due to forces inherent in the water itself. But we cannot yet state with any certainty that the same is true for the effects of ions of lower valency on the cataphoretic P.D. of solid particles.

We can say, however, that where the effects of an electrolyte on the cataphoretic P.D. differ with the nature of the solid particles the forces or conditions inherent in the solid particles must be responsible. The effects of this type of forces and of the forces inherent in the water may be superposed upon each other.

That some particles assume a positive charge in water in the absence of electrolytes can only be due to the chemical nature of the particle, whereby cations (including the hydrogen ions of the water) are more strongly attracted by the particle than the anions. It is noteworthy that the positive colloids mentioned in this paper are all electrolytes. Negative colloids may be electrolytes or non-electrolytes, but since gas bubbles are generally negatively charged, the charge of negative colloids need not be entirely due to a preferential attraction of anions by the solid particle but may be due partly or entirely to the forces inherent in the water as discussed in the preceding papers.[2]

It must be ascribed to forces inherent in the particle that the point of reversal of the sign of charge in the case of proteins coincides with the isoelectric point of the particle.

The considerable variation in the maximal cataphoretic P.D. with the chemical nature of the particle in solutions of $Na_4Fe(CN)_6$ and NaOH (as shown in Tables I and II) must also be ascribed to the influence of the particles themselves.

SUMMARY.

1. The effect of eight salts, NaCl, Na_2SO_4, $Na_4Fe(CN)_6$, $CaCl_2$, $LaCl_3$, $ThCl_4$, and basic and acid fuchsin on the cataphoretic P.D. between solid particles and aqueous solutions was measured near the

point of neutrality of water (pH 5.8). It was found that without the addition of electrolyte the cataphoretic P.D. between particles and water is very minute near the point of neutrality (pH 5.8), often less than 10 millivolts, if care is taken that the solutions are free from impurities. Particles which in the absence of salts have a positive charge in water near the point of neutrality (pH 5.8) are termed positive colloids and particles which have a negative charge under these conditions are termed negative colloids.

2. If care is taken that the addition of the salt does not change the hydrogen ion concentration of the solution (which in these experiments was generally pH 5.8) it can be said in general, that as long as the concentration of salts is not too high, the anions of the salt have the tendency to make the particles more negative (or less positive) and that cations have the opposite effect; and that both effects increase with the increasing valency of the ions. As soon as a maximal P.D. is reached, which varies for each salt and for each type of particles, a further addition of salt depresses the P.D. again. Aside from this general tendency the effects of salts on the P.D. are typically different for positive and negative colloids.

3. Negative colloids (collodion, mastic, Acheson's graphite, gold, and metal proteinates) are rendered more negative by low concentrations of salts with monovalent cation (*e.g.* Na) the higher the valency of the anion, though the difference in the maximal P.D. is slight for the monovalent Cl and the tetravalent $Fe(CN)_6$ ions. Low concentrations of $CaCl_2$ also make negative colloids more negative but the maximal P.D. is less than for NaCl; even $LaCl_3$ increases the P.D. of negative particles slightly in low concentrations. $ThCl_4$ and basic fuchsin, however, seem to make the negative particles positive even in very low concentrations.

4. Positive colloids (ferric hydroxide, calcium oxalate, casein chloride—the latter at pH 4.0) are practically not affected by NaCl, are rendered slightly negative by high concentrations of Na_2SO_4, and are rendered more negative by $Na_4Fe(CN)_6$ and acid dyes. Low concentrations of $CaCl_2$ and $LaCl_3$ increase the positive charge of the particles until a maximum is reached after which the addition of more salt depresses the P.D. again.

5. It is shown that alkalies (NaOH) act on the cataphoretic P.D. of both negative and positive particles as $Na_4Fe(CN)_6$ does at the point of neutrality.

6. Low concentrations of HCl raise the cataphoretic P.D. of particles of collodion, mastic, graphite, and gold until a maximum is reached, after which the P.D. is depressed by a further increase in the concentration of the acid. No reversal in the sign of charge of the particle occurs in the case of collodion, while if a reversal occurs in the case of mastic, gold, and graphite, the P.D. is never more than a few millivolts. When HCl changes the chemical nature of the colloid, *e.g.* when HCl is added to particles of amphoteric electrolytes like sodium gelatinate, a marked reversal will occur, on account of the transformation of the metal proteinate into a protein-acid salt.

7. A real reversal in the sign of charge of positive particles occurs, however, at neutrality if $Na_4Fe(CN)_6$ or an acid dye is added; and in the case of negative colloids when low concentrations of basic dyes or minute traces of $ThCl_4$ are added.

8. Flocculation of the suspensions by salts occurs when the cataphoretic P.D. reaches a critical value which is about 14 millivolts for particles of graphite, gold, or mastic or denatured egg albumin; while for collodion particles it was about 16 millivolts. A critical P.D. of about 15 millivolts was also observed by Northrop and De Kruif for the flocculation of certain bacteria.